SOME SURFACE AND FUNCTIONAL PROPERTIES OF THYMUS-DERIVED LYMPHOCYTES

by

Sylvia Mary Kirov

A thesis submitted for the
degree of
Doctor of Philosophy
at the
Australian National University

February 1975

TABLE OF CONTENTS

STATEMENT

The investigations described in this thesis constitute my own original work. Most were carried out by myself, but some in Chapters 5, 6 and 7, involving the Isopaque/Ficoll separation procedure were carried out jointly with Dr. C.R. Parish.

Sylvia Kirov.

Sylvia Mary Kirov
Department of Microbiology
John Curtin School of Medical Research
Australian National University.

ABBREVIATIONS

ATC	:	Activated thymus cells.
ATxBM	:	Adult thymectomized, irradiated, bone marrow reconstituted mice.
B cell	:	Bone marrow (bursa)-derived lymphocyte.
BSA	:	Bovine serum albumin.
C'	:	Complement.
Con A	:	Concanavalin A.
CSL	:	Commonwealth Serum Laboratories.
DBSS	:	Dulbecco's Balanced Salt Solution.
DNP	:	Dinitrophenyl.
DOC	:	Deoxycholate.
DTH	:	Delayed type hypersensitivity.
E	:	Erythrocytes.
E-POL	:	POL sensitized erythrocytes.
FCA	:	Freund's complete adjuvant.
FcRL	:	Fc receptor lymphocytes.'
FCS	:	Foetal calf serum.
FIA	:	Freund's incomplete adjuvant.
HCY	:	Haemocyanin.
HGG	:	Human gamma globulin.
HRBC	:	Horse red blood cells.
Ig	:	Immunoglobulin.
2ME	:	2 Mercapto-ethanol.
MON	:	Monomeric flagellin.
MLC	:	Mixed lymphocyte culture.
NP-40	:	Nonidet-P-40.
ORBC	:	Ox red blood cells.
PFC	:	Plaque forming cells.
PHA	:	Phytohaemagglutin.
POL	:	Polymeric flagellin.
RFC	:	Rosette forming cells.
SRBC	:	Sheep red blood cells.
T cell	:	Thymus-derived lymphocytes.
TDL	:	Thoracic duct lymphocytes.
WBC	:	White blood cells.

CHAPTER 1

General introduction : aims and outlines of thesis

1. INTRODUCTION:

This thesis is concerned with thymus-derived lymphocytes (T cells) and in particular the nature of their receptor for antigen and their role in cellular collaboration.

1.1 The T cell receptor

The reaction of antigen with lymphocytes is highly specific and is believed to involve special receptors on the surface of the lymphocyte. The chemical nature of these receptors on T cells is probably the most controversial issue in cellular immunology at present. The view that immunoglobulin (Ig) is the receptor, as it appears to be for bone-marrow (or bursa)-derived lymphocytes (B cells) has been challenged by the failure of many investigators to demonstrate Ig directly on the surface of T cells or to inhibit various T cell responses with anti-Ig reagents. The first part of this thesis is concerned with the presence and functional significance of Ig on T cells.

1.1.1 Immunoglobulin on T cells: Is it there? Does it have a functional significance?

In 1972, when the work described in this thesis was commenced, these questions were being vigorously debated. The most common method used to detect Ig on T cells, direct visualization by reaction of cells with labelled anti-Ig (e.g. with fluorescent chromophores, radioiodinated, hybridized with anti-viral antibody coupled with ferritin or enzyme conjugated - see Warner, 1974) led to the conclusion that there was very much less Ig on T cells than on B cells. (Raff, 1970a; Rabellino et al., 1971; Unanue et al., 1971; Lamelin et al., 1972; Matter et al., 1972; Nossal et al., 1972; Gonatas et al., 1972.) Sensitive methods (e.g. radioautography with long exposures) (Bankhurst and Warner, 1971; Nossal et al., 1972) revealed approximately 500 molecules of Ig on thymus cells compared to 20,000 or more molecules on B cells. Ig was more readily demonstrated on peripheral T cells in lymph node or spleen (Hämmerling and Rajewsky, 1971) or activated T cells, for example T cells from thoracic duct lymph of F_1 animals given parental thymus cells (T-TDL) (Bankhurst et al., 1971).

However, some workers found no significant difference between labelling cells in thymus and peripheral T cells (Grey et al., 1972a) or T-TDL (Nossal et al., 1972). Nevertheless, the theory developed that Ig was present on T cells but that it was somehow "buried" in the membrane and inaccessible to the anti-Ig reagents in resting T cells till, upon activation, it became exposed and was therefore more readily detected.

Support for this theory grew. Greaves (1970) had shown that splenic T cells could be prevented from binding SRBC by pretreatment with anti-Fab antiserum but not by anti-heavy chain sera. Later, however, Hogg and Greaves (1972) found that some anti-μ chain sera could inhibit the binding. These were shown to be sera directed against the "hinge" region determinants of the μ heavy chain. Other anti-μ chain sera or other anti-heavy chain sera were not effective at blocking rosette formation. These data implied that the IgM antigen receptor molecules on the T cell were only partially exposed. (Greaves and Hogg, 1971.) A similar interpretation was given by Marchalonis and coworkers (1972a, b, c; Marchalonis and Cone, 1973). They used a lactoperoxidase iodination technique to label cell surface proteins and then examined cell extracts for labelled Ig. They concluded that T cells had significant amounts of IgM on their outer surface, of the same order as on B cells. Electron microscopic studies of the outer membrane of T and B cells revealed a thick glycocalyx layer present on T cells but not nearly so obvious on B cells. (Santer et al., 1972) and this was taken as more evidence for the inaccessibility of T cell Ig on intact cells to anti-Ig reagents.

Evidence for a functional role for Ig on T cells came from the ability of anti-Ig sera to block antigen binding (Dwyer et al., 1972), and to block other expressions of T cell recognition, such as graft versus host reactions (GVH) (Mason and Warner, 1970; Greaves et al., 1969; Rouse and Warner, 1972), mixed lymphocyte reactions (MLC) (Greaves et al., 1971), delayed hypersensitivity (DTH) (Theis and Thorbecke, 1972), suicide by radioactive antigen (Basten et al., 1971; Cooper and Ada, 1972), and T-B co-operation (Lesley et al., 1971; Cheers et al., 1971). In addition, experiments by Feldmann and Basten (1972b) and Feldmann (1972c) characterized a specific

collaborative factor released from activated thymus cells (ATC) as an IgM-like molecule of size 7-8s. This provided perhaps the most telling argument that activated T cells possessed functionally active IgM and most probably had receptors that were essentially similar to those on B cells.

However, other groups had failed to find hidden or inaccessible Ig on the T cell membrane. Grey et al. (1972b) solubilized cells in detergent, urea-acetic acid or by freeze-thawing and assayed cell extracts for Ig using a quantitative inhibition assay. They concluded that T cells did not have large amounts of Ig bound on the cell membrane. A similar conclusion was reached by Vitetta et al. (1972) using the same detection technique as Marchalonis and later by a third group. (Lisowska - Bernstein et al., 1973). By adding known numbers of B cells to thymocyte suspensions Vitetta et al. (1972) calculated that if thymocytes had any IgM, it would be less than 250 molecules per cell. This calculation was close to other estimates determined by Nossal et al. (1972) and Grey et al. (1972a) using different surface labelling techniques. Lisowska - Bernstein et al. (1973) showed that Ig synthesis by activated thymus cell populations was due to contamination of these cells by IgM containing plasma cells.

There were also reports of unsuccessful attempts to block T cell function with anti-Ig sera. Probably the most thorough of these was the work on GVH reactions in chickens by Simonsen's group who concluded that Ig was not the receptor for this type of recognition. (Crone et al., 1972.) Other research groups also failed to block GVH reactions (Ivanyi et al., 1970), or cytotoxic T cells, derived from the thymus, in either the sensitization (Feldman et al., 1972;) or the effector stage (Chapuis and Brunner, 1971; Feldman et al., 1972) with anti-Ig. Some of these investigators, however, (Crone et al., 1972; Feldman et al., 1972) showed that though anti-Ig sera were ineffective at blocking the T cell function, antisera directed at other membrane components, such as histocompatibility antigens, were effective. They postulated that the positive results obtained by other groups may be due to contamination of the anti-Ig reagents with antibody directed against membrane components other than Ig or that the anti-Ig

reagents acted via steric hindrance.

Another controversial issue concerning Ig on T cells was whether the amounts detected were in fact synthesized by the T cells or acquired passively. Nobody had definitely demonstrated that pure T cells could synthesize Ig. Surface Ig had been detected on some malignant cell lines, apparently of T cell origin (Marchalonis et al., 1972a) and synthesis of Ig by these cell lines had been reported (Harris et al., 1972), but malignant cells cannot be regarded as normal T cells. In addition, other evidence had been published which suggested that tumour cells could acquire Ig cytophilically (Grey et al., 1972b).

Some evidence that Ig was not acquired passively by T cells came from the work of Basten et al. (1972). Using the sensitive radioautographic technique, they showed that ^{125}I labelled antigen-antibody complexes did not bind to T cells from several sources, including activated thoracic duct cells, although they bound to B cells. They concluded that B, but not T cells, possess a receptor for the Fc portion of Ig. Cline et al. (1972) also found activated T-TDL unable to form rosettes with antibody coated erythrocytes. However, other workers, (Yoshida and Andersson, 1972) detected 2-5% Fc rosettes in normal thymus and about 70% of T cells activated to histocompatibility antigens from spleen or lymph nodes gave Fc rosettes. This rosette formation could not be significantly inhibited by free mouse Ig but could be inhibited by mouse antigen-antibody complexes. They concluded that antigen activated mouse T cells did have receptors for antigen complexed Ig. Thus, whether T cells possessed a receptor for Fc was still being debated. There had been no reports of functionally active T cells possessing an Fc receptor.

Some of these controversial issues concerning Ig on T cells and its functional significance were considered (see 1.3) and results are reported in Chapters 2 and 5.

1.2 Role of the T cell in cellular collaboration

The second part of this thesis is concerned with collaboration between T and B cells in the induction of antibody synthesis. Collaboration was first suggested by the

experiments of Claman et al. (1966) in which it was shown that irradiated mice given both thymus and bone marrow cells made a far greater primary response to SRBC than recipients which received either thymus or bone marrow cells alone. Subsequent work (Davies et al., 1967; Miller and Mitchell, 1968; Nossal et al., 1968) clearly demonstrated that T cells had a "helper" role and B cells produced the antibody. Co-operation between T and B lymphocytes has now been observed in vitro as well as in vivo, in both primary and secondary humoral responses and has been demonstrated for a variety of antigens. Hapten-carrier antigens have been studied most. The two antigenic components, hapten and carrier, are thought to represent separate sites of functional recognition by T and B lymphocytes, with haptens binding to Ig receptors on B cells and the carrier being recognized by T cells. (Mitchison et al., 1970; Mitchison, 1971.) This phenomenon is known as the "carrier" effect. Carrier effects are not confined to hapten-protein conjugates. They can be demonstrated for example, with the sub-units of protein molecules (Rajewsky et al., 1967) or with separate determinants on erythrocytes. (Cunningham and Sercarz, 1971). Hapten carrier antigens have provided a valuable tool for looking at T-B collaboration because the two functional antigenic determinants can be separately identified. Collaboration between T and B cells has been extensively discussed in recent reviews. (Basten and Howard, 1973; Bullock and Moller, 1974; Claman and Chaperon, 1969; Davies, 1969; Feldmann and Nossal, 1972; Katz and Benacerraf, 1972; Playfair, 1971; Playfair, 1973; Miller, 1972; Miller and Mitchell, 1969; Miller et al., 1971a; Mitchell, 1974.) However, the mechanisms by which T and B cells collaborate are not definitely known. Models that have been proposed may be broadly classified into the Contact models and the Factor models.

Contact models propose:-

a) that antigen is more efficient at activating B cells when exposed as an ordered array on the T cell surface - "local concentration hypothesis" (Mitchison, 1969; Möller, 1970).

or

b) that as T cells recognize foreign antigen on the

B cell surface they release non-specific activating factors which stimulate B cells directly - "T cell surveillance hypothesis" (Kreth and Williamson, 1971).

or

c) that cell-cell contact via an antigen bridge allows a surface membrane interaction between T and B cells resulting in B cell activation. (Katz et al., 1973a)

Evidence cited in favour of these contact models is that:-

a) chemical linkage between the hapten and the carrier determinant is required for the anti-hapten response; (Mitchison, 1971; Kettman and Dutton, 1971).

b) T independent antigens are antigens which have repeating antigenic determinants. For example, the response to polymeric flagellin (POL) is T independent in mice, whereas the response to monomeric flagellin (MON) is dependent on the presence of MON primed T cells. (Feldmann and Basten, 1971).

c) the "allogeneic effect", (i.e. abnormal induction of B cells by allogeneic T cells - Katz et al., 1971a, b) is only obtained when hapten primed B memory cells represent the targets for the allogeneic aggressor cells. No activation occurs when the precursor cells for anti-hapten antibody production are "innocent bystanders", i.e. part of the allogeneic population (Rajewsky et al., 1972)

d) T-B collaboration is restricted to histocompatible cells. (Katz et al. 1973 a and b).

However, against the contact models are that:-

a) the T cell does not appear to have merely the passive role of antigen presentation. Feldmann and Basten (1972a) showed that protein and RNA synthesis were required for collaboration. In addition, other cell types coated with antigen or

antigen coated inert particles or non-reactive T cells coated with antigen could not collaborate with B cells. (Unanue, 1970; Katz et al., 1970; Miller et al., 1971b).

b) two relatively rare specific T and B cells would need to contact each other and T cells have been shown to enhance the response of antigens *in vivo*, even when B cells outnumber them by a ratio of 1000:1 (Rajewsky et al., 1972).

c) T cell co-operation with B cells has been shown to occur across a cell impermeable membrane (Feldmann and Basten, 1972b).

These latter objections have led to the Factor models for T-B collaboration. *Factor models* propose:-

a) T cells upon contact with antigen are activated to secrete non-specific B cell mitogenic products;
or

b) T cells upon contact with antigen secrete antigen specific activating substances;
or

c) both a) and b) occur.

Production of non-specific T cell replacing factors has been reported by many workers (e.g. Davies et al., 1967; Kennedy et al., 1970; Ekapaha-Mensah and Kennedy, 1971; Dutton et al., 1971; Doria et al., 1972; Rubin and Coons, 1972a, b; Rubin et al., 1973; Schimpl and Wecker, 1972, 1973; Britton, 1972; Gorczynski et al., 1972a, b, 1973; Feldmann and Basten, 1972c; Sjöberg et al., 1972; Rosenthal et al., 1973; Watson, 1973; Gisler et al., 1973; Geha et al., 1973; Waldmann and Munro, 1973, 1974; Waldmann et al., 1973; Armerding and Katz, 1974). Factors were obtained from primed T cells cultured with the inducing antigen, (e.g. Rubin and Coons, 1972a, b; Waldmann and Munro 1973, 1974; Waldmann et al., 1973; Geha et al., 1973; Gisler et al., 1973), or from allogeneic cell supernatants, (e.g. Schimpl and Wecker 1972, 1973; Gorczynski et al., 1972a, b, 1973; Feldmann and Basten, 1972c; Armerding and Katz, 1974) or from mitogen activated T cells (Sjöberg et al., 1972) and even from unprimed spleen cells cultured with SRBC (Doria et al., 1972). However, in most reports the evidence

for T cell production of the factor was not conclusive. Waldmann and Munro (1973) showed that addition of adherent cells to activated thymus cells led to a dramatic increase in production of their T cell replacing factor. Thus the possibility existed that T cells activated by antigen may cause macrophages to liberate a T cell replacing factor. In addition, the site of action of the factor is an important consideration. Most workers studied the effect of the factor in stimulating the response of ATxBM spleen cells or normal spleen cells cultured with antigen. Thus whether the factor was acting directly on B cells or was causing expansion of a small residue of T cells or T cell precursors which could then help in the response was unknown. Schimpl and Wecker (1972) and Askonas, Schimpl and Wecker (1974) demonstrated the existence of two factors - 1) a T cell expanding factor (TEF) which stimulated the proliferation of T cells and thus led to functional replenishment of the T cell pool, in systems partially deprived of T cells, and 2) a T cell replacing factor (TRF) which actually replaced T cells in their co-operation with B cells. They used anti-θ treated nude (congenitally athymic) mouse spleen cells to demonstrate that TRF acted as a differentiation signal favoring Ig production by an antigen activated B cell. They showed that reduction of T cells by treatment with anti-θ serum abolished TRF production whereas reducing the number of macrophages of a mixed lymphocyte culture after 14h (before TRF appearance) did not affect the yield of TRF.

Thus, non-specific factors, presumably produced from T cells, can replace the collaborative role of T cells in vitro. However, it must be postulated that these factors are short range in action to explain, for example, the requirement for hapten-carrier linkage for an anti-hapten response, and the in vivo experiment of Rajewsky et al. (1972) where there was no antibody production when the antibody precursor cells were "innocent bystanders" during a GVH reaction.

Specific T cell replacing factors have also been reported (Feldmann and Basten, 1972b, c and d; Yu and Gordon, 1973; Gisler et al., 1973; Taussig, 1974; Taussig and Munro, 1974). In most cases these have been produced by activated thymus cells (ATC) i.e. cells obtained from spleens of

irradiated recipients given thymus cells and antigen 7 days previously. Two groups have attempted to characterize these specific factors and disagree as to their nature. Feldmann (1972c) and Feldmann et al. (1973) showed that their factor was similar to monomeric IgM complexed with antigen. It was cytophilic for macrophages and formed a functional immunogen on their surface which could activate B cells of the appropriate specificity. Taussig and Munro (1974), however, claimed that their factor could not be removed by a sheep anti-mouse Ig absorbent, but was removed completely by congenically raised anti-H-2 antibodies. This coincided with a similar characterization by Tada et al. (1973) of a specific T cell suppressive factor and is consistent with there being H-2 requirements for T-B collaboration. (Katz et al., 1973b), unlike Feldmann's factor which would seem to allow effective co-operation between allogeneic cells.

Active synthesis of specific collaborative factors by T cells has not been demonstrated, and the possibility that some may be from B cells contaminating the ATC preparations or B cell products on the surface of activated thymus cells (Hunt and Williams, 1974; Sprent and Hudson, 1973; Hudson et al., 1974) must be considered. Antibody mediated enhancement of an immune response is a well established phenomenon (e.g. see Henry and Jerne, 1968; Dennert, 1971; Pincus et al., 1971, 1973; Rubin 1972a, b; Taussig and Lachmann, 1972; Brody et al., 1967; Walker and Siskind, 1968; McBride and Schierman, 1970, 1973; Terres and Morrison, 1967; Murgita and Vas, 1972; Janeway and Paul, 1973; Janeway, 1973), but the mechanism by which specific antibody can augment antibody responses is not known. Recently, Playfair et al. (1974) have suggested that the receptor for antigen on T helper cells is adsorbed antibody, and that antibody given to animals passively augments antibody responses by arming T cells with specific receptors. They claim this could explain some of the controversy regarding Ig on T cells, discussed in 1.1. J.F.A.P. Miller has also suggested that B cells present in the ATC population are responsible for production of the specific collaborative factor, (personal communication).

Hence it is clear that there are still many questions regarding mechanisms of T-B collaboration and the nature, role and *in vivo* significance of non-specific and specific T cell replacing factors. Some questions are answered and others raised by the data presented in the second part of this thesis, where collaboration between cells in an anti-hapten response *in vitro* were examined.

1.3 Outlines of Thesis

Initially, thymus cells were examined for surface Ig by reacting them with a purified ^{125}I-labelled anti-light chain antibody. This reagent was one which had been previously shown to block suicide of the DTH reaction to flagellin by ^{125}I-labelled flagellin (Cooper and Ada, 1972). Thymus cells were also examined for total Ig by solubilizing the cells and assaying the Ig present in cell extracts by a quantitative microprecipitin technique (Chapter 2). Chapter 3 describes a plaque assay for measuring anti-DNP plaque-forming cells and characterization of *in vitro* culture systems for obtaining good anti-hapten responses to DNP-haemocyanin (DNP-HCY), DNP-human gamma globulin (DNP-HGG) and DNP-flagellin (DNP-MON). During characterization of these responses, spleen cells from the congenitally athymic "nude" mouse mutant were used as negative controls to establish the T cell dependent nature of the responses. However, nude spleen cells gave excellent responses to DNP-MON. This discrepancy was resolved when nude mice were shown to have a small proportion of functionally active T cells able to collaborate in the anti-hapten response and give a delayed type hypersensitivity reaction to MON injected into the hind footpad (Chapter 4).

In Chapter 5, a method for obtaining pure populations of mouse T and B cells by fractionating cells on Isopaque/Ficoll was adapted in collaboration with C.R. Parish, from a cell separation procedure he had worked out for rat lymphocytes and was characterized with the techniques described in Chapters 2 and 3. Also, the procedure was modified to detect Fc rosetting cells and used to see whether Fc receptors were present on any functional T cells.

The *in vitro* techniques and cell separation procedure were combined to study T-B collaboration to DNP-MON and the role of soluble factors (Chapters 6 and 7).

Results of these studies are discussed in relation to those of other investigators in Chapter 8.

1.4 Communications

Much of the work presented in this thesis and other collaborative work not presented here has been published and presented at meetings as follows:-

1. Kirov, S.M. and Ada, G.L.
   Immunoglobulins on thymus cells: reaction and quantitation by microprecipitin inhibition with cell extracts.
   Scand. J. Immunol. 1974. 3 : 85

2. Kirov, S.M., Ey, P.L. and Ada, G.L.
   Immunoglobulins associated with mouse lymphocytes.
   Abstr. Ann. Meet. Aust. Soc. Immunol., Sydney, 1972. p. 17.

3. Kirov, S.M.
   An anti-theta sensitive hapten-carrier response in nude mice.
   Eur. J. Immunol. 1974. 4 : 739.

4. Parish, C.R., Kirov, S.M. Bowern, N. and Blanden, R.V.
   A one-step procedure for separating mouse T and B lymphocytes.
   Eur. J. Immunol. 1974. In press.

5. Kirov, S.M. and Parish, C.R.
   A specific T cell replacing factor. I. Detection and evidence that B cells are required for its production.
   Submitted manuscript.

6. Kirov, S.M. and Parish, C.R.
   A specific T cell replacing factor. II. A role for carrier specific B cells and macrophages in cellular collaboration.
   Submitted manuscript.

7. Kirov, S.M. and Parish, C.R.
A role for the carrier specific B cell in cellular collaboration.
Abstr. Ann. Meet. Aust. Soc. Immunol.
Canberra, 1974, p. 49.

8. Kirov, S.M. and Globerson, A.
Uncommitted stem cells in the embryonic liver.
Abstr. Ann. Meet. Aust. Soc. Immunol. Canberra, 1974.
p. 39.

CHAPTER 2

Immunoglobulins on thymus cells: Reaction with anti-light chain antibody and quantitation by microprecipitin inhibition with cell extracts.

2.1 INTRODUCTION

The aim of the experiments reported in this chapter was to examine thymus cells for surface immunoglobulin and try to quantitate this. The reagent used in these studies was an anti-light chain immunoglobulin which could block inactivation of T cell function (Cooper and Ada, 1972). First the amount of reagent which bound to cells was assayed both by total counts and by examining cells individually by radio-autography. Second, thymus cells were compared with B cells by assaying the total Ig present in extracts of the two classes of cells.

2.2 MATERIALS AND METHODS

2.2.1 Animals

Unless otherwise stated, CBA/J mice were used in the experiments described in this chapter.
CBA/J, CBA/H, Balb c, (CBA x C57 Bl)F_1 were obtained from breeding colonies in this School.
ATxBM (Adult thymectomized, irradiated, bone marrow reconstituted) mice were thymectomized at 7 weeks of age, lethally irradiated (850 rads) two weeks later and reconstituted by i.v. injection of 2×10^7 bone marrow cells.

2.2.2 Sources of lymphoid cells

Thymus and spleen cell suspensions were prepared by cutting these organs into small pieces with scissors and gently teasing the pieces through a stainless steel sieve into cold Dulbecco's balanced salt solution (DBSS) (Dulbecco et al., 1954) containing 10% foetal calf serum (FCS). Special care was taken when removing thymuses to have a minimum of blood contamination and to exclude nearby lymph nodes. All surrounding fatty areas were cleaned from the thymus routinely before cell suspensions were prepared. Bone marrow cells were obtained by flushing out the femurs and tibias with medium using a syringe and 26G x ½" needle. Cell clumps were broken up by gently aspirating the cells through the syringe.

White blood cells (WBC) were obtained from whole blood using a ficoll flotation method described by Noble et al. (1968). All cell suspensions were thoroughly washed 3 times in medium and counted using a haemocytometer. Viability was judged by trypan blue dye (0.05%) exclusion and in most

cases was greater than 95%.

2.2.3 Immunoglobulin reagents

2.2.3.1 Rabbit anti-CBA mouse light chain globulin (anti-mouse L chain Ig)

This antibody was prepared by Mrs. B.J. Howlett and full details are given elsewhere (Cooper and Ada, 1972). Briefly, serum globulins from rabbits made hyperimmune to a Balb c mouse myeloma IgA were recycled through a sepharose column containing mouse IgG until most anti-L-chain activity had been adsorbed. The antibody was then eluted from the column with 1N propionic acid and neutralized by dialysis against DBSS.

2.2.3.2 Rabbit anti-sheep light chain globulin (anti-sheep L chain Ig)

This reagent was kindly donated by Dr. P.L. Ey. It was prepared similarly to the anti-mouse reagent. Details of its preparation and specificity are given elsewhere (Ey, 1973).

2.2.3.3 Anti-θ globulin

The reagent used was a concentrate of an ammonium sulphate (50%) precipitate of an anti-θ ascitic fluid. The latter was prepared in AKR/J mice which were given 10 intraperitoneal injections of 5-10 x 10^7 CBA thymocytes at weekly intervals. The first injection was given with 1 x 10^9 B. pertussis organisms (C.S.L., Melbourne). Three days before the final dose of thymocytes, the mice were injected intraperitoneally with 0.2 ml of a 10% suspension of sarcoma 180 cells in saline (Tikasingh et al., 1966). The ascitic fluid was harvested 7 days after the final thymocyte injection. Unimmunized AKR/J mice were used as donors of control ascitic fluid and a similar ammonium sulphate precipitate of this used as a control for the anti-θ globulin. Undiluted, the anti-θ globulin had a cytotoxicity of >98% for thymocytes.

2.3.2.4 Anti-B-anti-macrophage

The preparation and specificity of this reagent, (an antiserum from rabbits immunized with thioglycollate induced macrophages) kindly donated by Dr. R.V. Blanden, has been described elsewhere (Blanden and Langman, 1972). After

adsorption with 5 x 10^8 CBA thymocytes/ml it was found to be cytotoxic for B but not T cells.

2.3.2.5 Anti-heavy chain sera

Anti-μ and anti-γ chain sera, prepared by P.L. Ey (Ey, 1973), were obtained from the hyperimmune sera of rabbits injected with purified mouse (CBA) IgM and IgG respectively. Anti-light chain activity was removed by passing the sera through sepharose-mouse IgG and IgM columns. The specificity of these reagents for heavy chains was shown by microprecipitin tests.

2.2.4 Labelling of the anti-light chain reagent with ^{125}I

The anti-light chain reagent was labelled with carrier-free [^{125}I] iodide (IMS3: The Radiochemical Centre, Amersham, U.K.) by direct oxidation with chloramine-T (Byrt and Ada, 1969). Labelling efficiencies were usually 70-90% and the specific activity of the protein was in the range 30-40 μC/μg protein. For radioactive anti-light chain suicide the specific activity was in the range 150-200 μC/μg.

2.2.5 Reaction of cells with radioactive protein

Cells were incubated (30 min , 0°C) at a concentration of 2 x 10^7 cells/0.2 ml of DBSS containing 10% FCS and 15mM sodium azide, with ^{125}I -anti-light chain IgG, 200 ngs being the "standard dose", but this varied in some experiments from 180-250 ngs . The cells were then washed by centrifuging through 2 gradients of FCS to remove unreacted anti-light chain Ig and the final cell pellet resuspended in a drop of FCS and smeared onto gelatin-coated slides for radioautography. The cell smears were fixed in methanol or acid methanol.

2.2.6 Radioautographic procedure

Full details of this technique are described by Byrt and Ada, (1969). Briefly, fixed slides were dipped in Kodak NTB-2 photographic emulsion, dried vertically, boxed in light-proof boxes containing "drierite" and stored at 4°C for 2-5 days when they were developed (Kodak D-19) and stained

with 5% Giemsa, mounted in DPX mounting medium and scanned for labelled cells.

Between 50000-100000 cells were scanned. Labelled cells were classified into four categories according to the number of grains associated with them:-
'L': <25 grains, 'M': 25-50 grains, 'H': >50 grains, 'HH': "hedgehog" - cell obscured by density of grains. The criterion for a labelled cell was that there were at least ten grains above background level, which in most cases was very low (2-3 grains per cell area).

2.2.7 Carbon-black visualization of parathymic lymph nodes

Pelikan black ink was centrifuged for 15 min at 2000 g and the supernatant diluted twofold with DBSS, and 0.2 ml injected intraperitoneally into mice 30 min before they were killed and thymuses removed.

2.2.8 Cortisone treatment

Mice were injected subcutaneously with 5 mg (0.2 ml) of cortisone acetate (Rousel, England) 24 hours before thymuses were removed.

2.2.9 Cytotoxicity Assay

Lymphoid cells were incubated (30 min , 37°C) at a concentration of 4×10^6 cells/0.2 ml with dilutions of the antiserum under test. They were then washed twice, resuspended in 0.2 ml of a twofold dilution of reconstituted guinea pig complement (CSL, Melbourne, Australia). The complement had been adsorbed previously on agarose (80 mg/ml complement) for 1 hour at 0°C and incubated for a further 30 min at 37°C. After this incubation the tubes were placed in ice and 0.2 ml ice-cold Trypan blue (0.1%) added. Aliquots of cells were counted for viability immediately in a haemocytometer.

2.2.10 Treatment of anti-light chain binding cells with various antisera and complement

Basically the same procedure as above was used, but the initial incubation with the antisera was done for one hour at 0°C in order to minimize the risk of loss of label from the cells at 37°C. The 30 min incubation with C' was carried

out at 37°C, but the C' was diluted twofold with medium containing azide.

2.2.11 Nigrosin staining technique for differentiation of live and dead cells

This technique was adapted from that described by Claesson et al. (1969). Cells (2×10^7) were resuspended in 0.1 ml chilled 0.9% sodium chloride in 0.25M sucrose and 0.1 ml 0.1% nigrosin in isotonic saline was added. After 10 min at 4°C the mixture was centrifuged through an FCS gradient and the cell pellet smeared for radioautography as usual. Slides were fixed in methanol. The staining procedure withstood the radioautographic procedure. The Feulgen counter stain, suggested by Claesson et al. was found to be unsatisfactory for radioautographs, as many of the silver grains dissolved during the HCl digestion. A one minute counter stain in Giemsa proved satisfactory - live cells staining a very pale blue, dead cells a dark purplish-blue.

2.2.12 Microprecipitin inhibition technique

Known numbers of lymphoid cells, ranging from 2-5 x 10^8 cells were solubilized in 0.5 ml 5% NP-40 containing 5% FCS and 0.1% azide at 0°C for more than one hour with occasional vortexing. They were spun for 15 minutes at 2500 g at 4°C and the supernatants used in microprecipitin inhibition assays. The diluent used also contained 5% NP-40, 5% FCS and 0.1% azide. Doubling dilutions of each cell extract and known IgM and IgG standards were made over an appropriate range to yield a final volume of 100µl. Both IgM and IgG were assayed in each cell extract. Appropriate amounts of specific anti-µ or anti-γ sera (20µl) were added to the cell extract dilutions, and kept at 4°C overnight with intermittent shaking. Samples (50µl) of the mixtures were then assayed in a microprecipitin test.

^{125}I -IgG or ^{125}I -IgM, (5ng amounts in 0.1M Tris HCl pH 7.2, 10% NRS, 15mM azide) in a final volume of 50µl were added to the inhibitor mixtures. The contents of the tubes were mixed thoroughly, incubated for 3 hours at 37°C and kept overnight at 4°C. They were then centrifuged (11000 g for 1 hour), and 50µl of supernatant counted in a gamma spectrometer.

The amount of IgM and IgG present in the cell extract dilutions could be judged by comparison with the known IgM and IgG standards at the 50% end-point. From this an estimate of the average amount of Ig present per cell could be gained.

2.2.13 Enumeration of plaque-forming cells

Anti-SRBC PFC were counted using the technique of Cunningham and Szenberg (1968). To detect anti-DNP PFC, SRBC were coated with dinitrophenylated rabbit anti-SRBC Fab, as described by Strausbauch et al. (1970) and in Chapter 3.

2.3 RESULTS

2.3.1 Anti-light chain binding cells in different tissues - evidence for specificity

In the first set of experiments, labelled anti-mouse light chain immunoglobulin was reacted with cells from bone marrow, spleen and thymus.

The results were expressed, both as counts of radioactivity associated with the cell pellet after the reaction, and as the proportion of cells labelled. There were consistently gross differences in both respects among these three different cell populations. In the experiment reported in Table 2.1, 40-50% of spleen cells, 10-15% of bone marrow cells and less than 1% of thymus cells bound anti-mouse light chain Ig.

The cell populations could be further distinguished by the proportion of cells in different categories when this was plotted according to the number of grains associated with cells (see Figure 2.1). Bone marrow and thymus showed a rather similar profile in which there was about the same proportion of heavy to lightly labelled cells; this pattern was different from that shown by spleen where there was a much higher proportion of lightly labelled cells.

As a control for the specificity of this reaction, cells were treated under the same conditions with labelled anti-sheep light-chain Ig. Although radioactivity was still found with the cell pellet, negligible numbers of labelled cells were detected. As a further test of specificity, cells were pre-exposed to an excess (40x) of either unlabelled anti-

Table 2.1: Anti-light chain binding cells in different tissues - evidence for specificity

Anti-light chain reagent a)	Tissue	Percentage bound (Bulk counts)	Percentage of labelled cells (Radioautography) b)
^{125}I-anti-mouse	B.M. c)	2.0	13
	Spl	6.7	44
	Thy	0.3	0.1
^{125}I-anti-sheep	B.M.	0.5	<0.3
	Spl	0.9	<0.3
	Thy	0.2	<0.002
Unlabelled Anti-mouse + ^{125}I-anti-mouse	B.M.	0.7	6
	Spl	4.7	38
	Thy	0.1	<0.002
Unlabelled Anti-sheep + ^{125}I-anti-mouse	B.M.	2.1	11
	Spl	6.4	41
	Thy	0.2	0.08

a) 250ng specific activity = approx. 30 µc/µg.

b) Radioautographs were exposed for 3 days.

c) B.M.: bone marrow, Spl.: spleen, Thy: thymus.

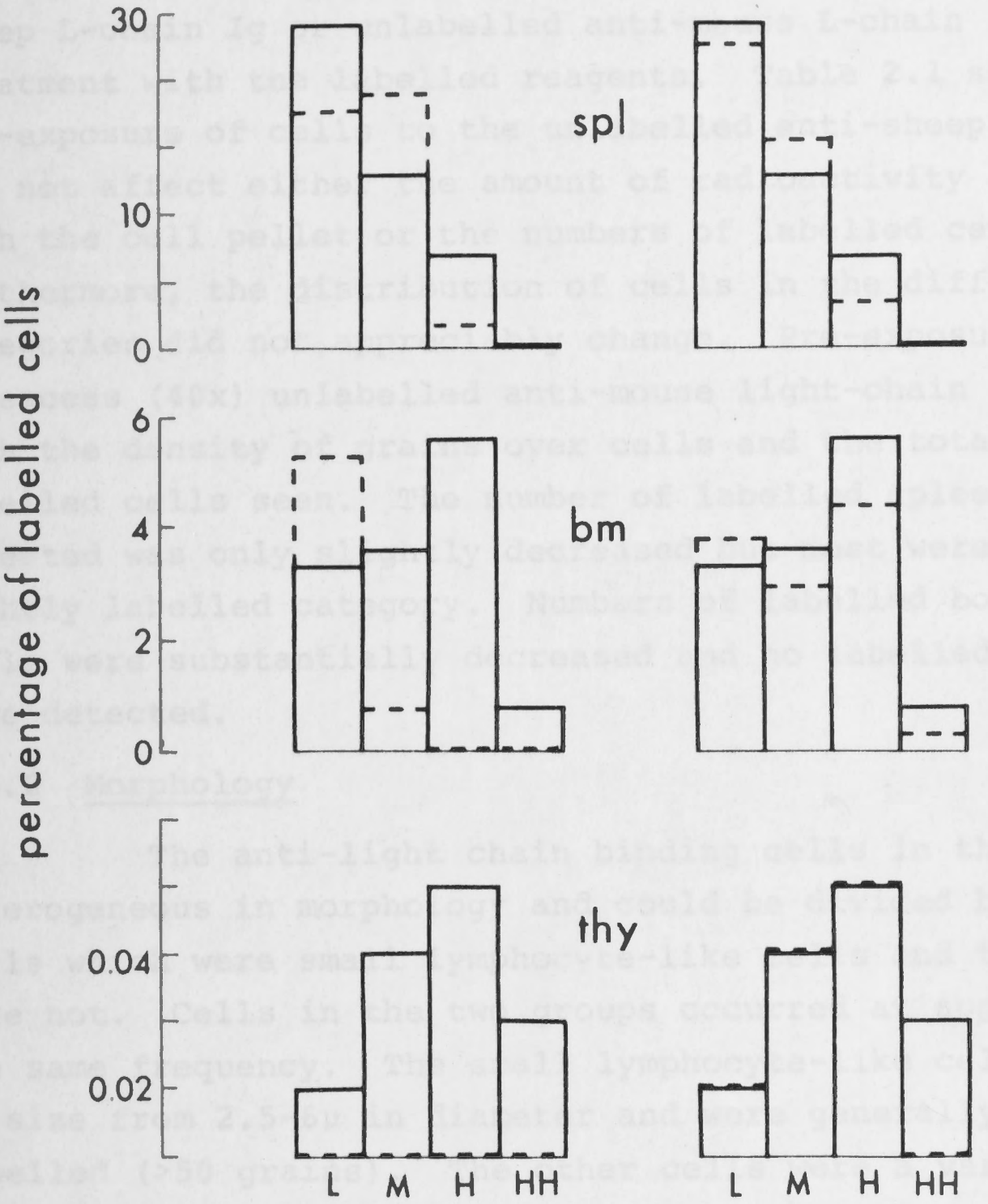


Figure 2.1 Evidence for specificity of anti-light chain binding.

The solid lines show the proportion of anti-CBA light chain binding cells of different grain distribution in the tissues examined. The dotted lines show the effect of pre-exposure to cold anti-mouse light chain (left) or cold anti-sheep light chain (right) on these anti-light chain binding cells.
'L' : < 25 grains; 'M' : 25-50 grains; 'H' : > 50 grains;
'HH' : "hedgehog" - cell obscured by density of grains.

sheep L-chain Ig or unlabelled anti-mouse L-chain Ig before treatment with the labelled reagents. Table 2.1 shows that pre-exposure of cells to the unlabelled anti-sheep reagent did not affect either the amount of radioactivity associated with the cell pellet or the numbers of labelled cells found. Furthermore, the distribution of cells in the different grain categories did not appreciably change. Pre-exposure of cells to excess (40x) unlabelled anti-mouse light-chain decreased both the density of grains over cells and the total numbers of labelled cells seen. The number of labelled spleen cells detected was only slightly decreased but most were now in the lightly labelled category. Numbers of labelled bone marrow cells were substantially decreased and no labelled thymus cells were detected.

2.3.2 Morphology

The anti-light chain binding cells in the thymus were heterogeneous in morphology and could be divided broadly into cells which were small lymphocyte-like cells and those which were not. Cells in the two groups occurred at approximately the same frequency. The small lymphocyte-like cells ranged in size from 2.5-6μ in diameter and were generally heavily labelled (>50 grains). The other cells were a variable group, often 10μ and greater in diameter, with varying proportions of basophilic cytoplasm. They were generally more lightly labelled. (Figure 2.2.)

In contrast to the variety of morphological types found in thymus, all the binding cells in bone marrow were about 5-6μ in diameter and many were heavily labelled. They were very similar in appearance to the heavily binding cells in the thymus. In spleen, most of the labelled cells were typical small lymphocytes with diameters 5μ and upwards, and some had a visible rim of cytoplasm. The binding cells in blood were relatively uniform being typical lymphocytes approximately 7.5μ in diameter; most were heavily labelled. (Figures 2.3 and 2.4.)

2.3.3 Nature of the binding cells found in thymus

In view of the very small proportion of cells which reacted with the anti-mouse light-chain Ig the first question

Figure 2.2

Anti-light chain binding cells in the thymus.

(a) small (<6μ), heavily labelled lymphocytes.

(b) larger (6-12μ) more lightly labelled cells.

(c), (d), (e) large cells (6-14μ) of varying morphology generally lightly labelled.

Scale in microns.

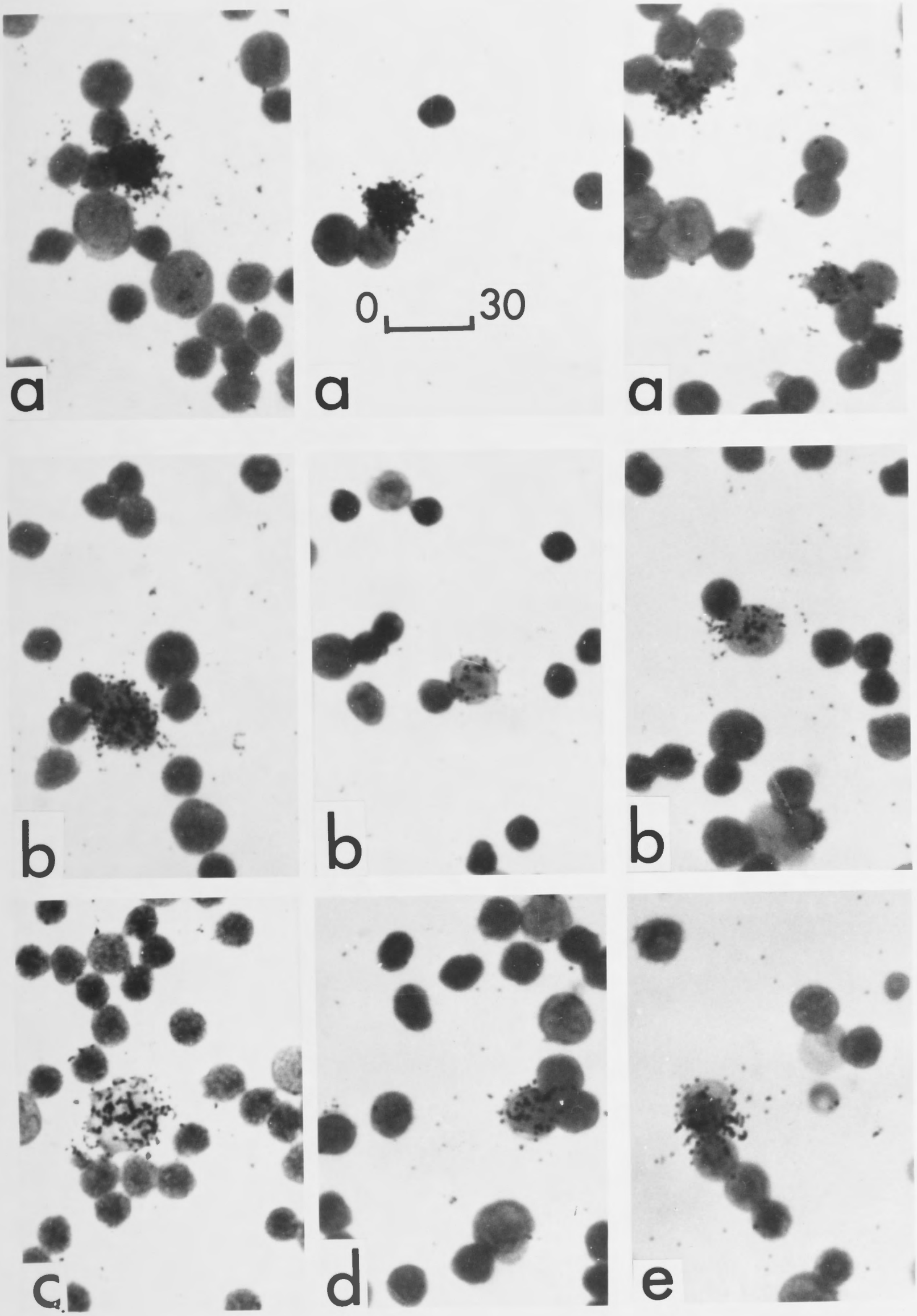

0
30
a
a
a
b
b
b
c
d
e

Figure 2.3

Anti-light chain binding cells in

(a) spleen
(b) thymus
(c) blood
(d) bone marrow.

Anti-light chain binding was carried out under "standard" conditions, described in the text and radioautographs exposed for 24 hours.

Scale in microns.

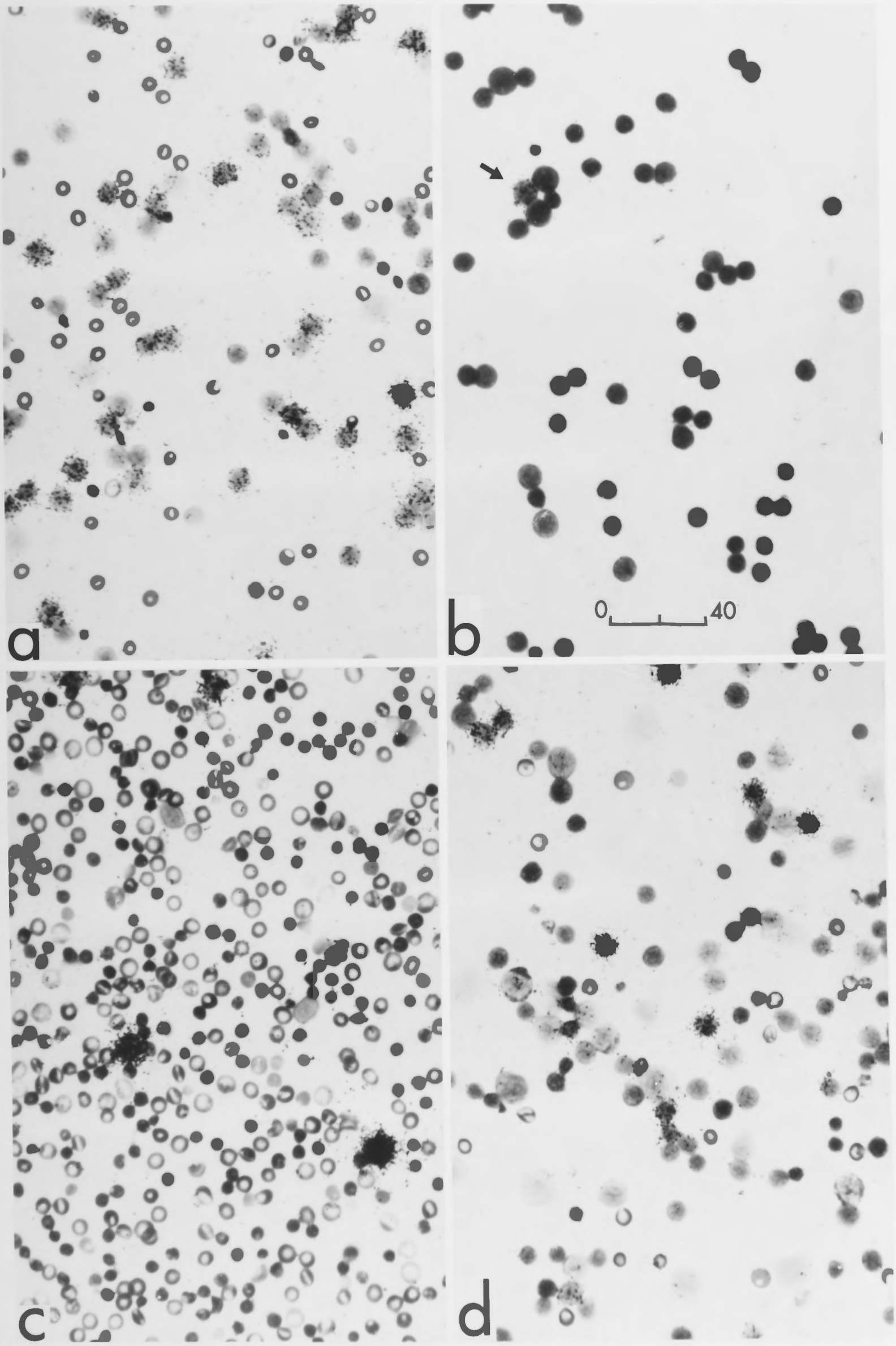

a
b
0
40
c
d

Figure 2.4

Higher magnification of anti-light chain binding cells in

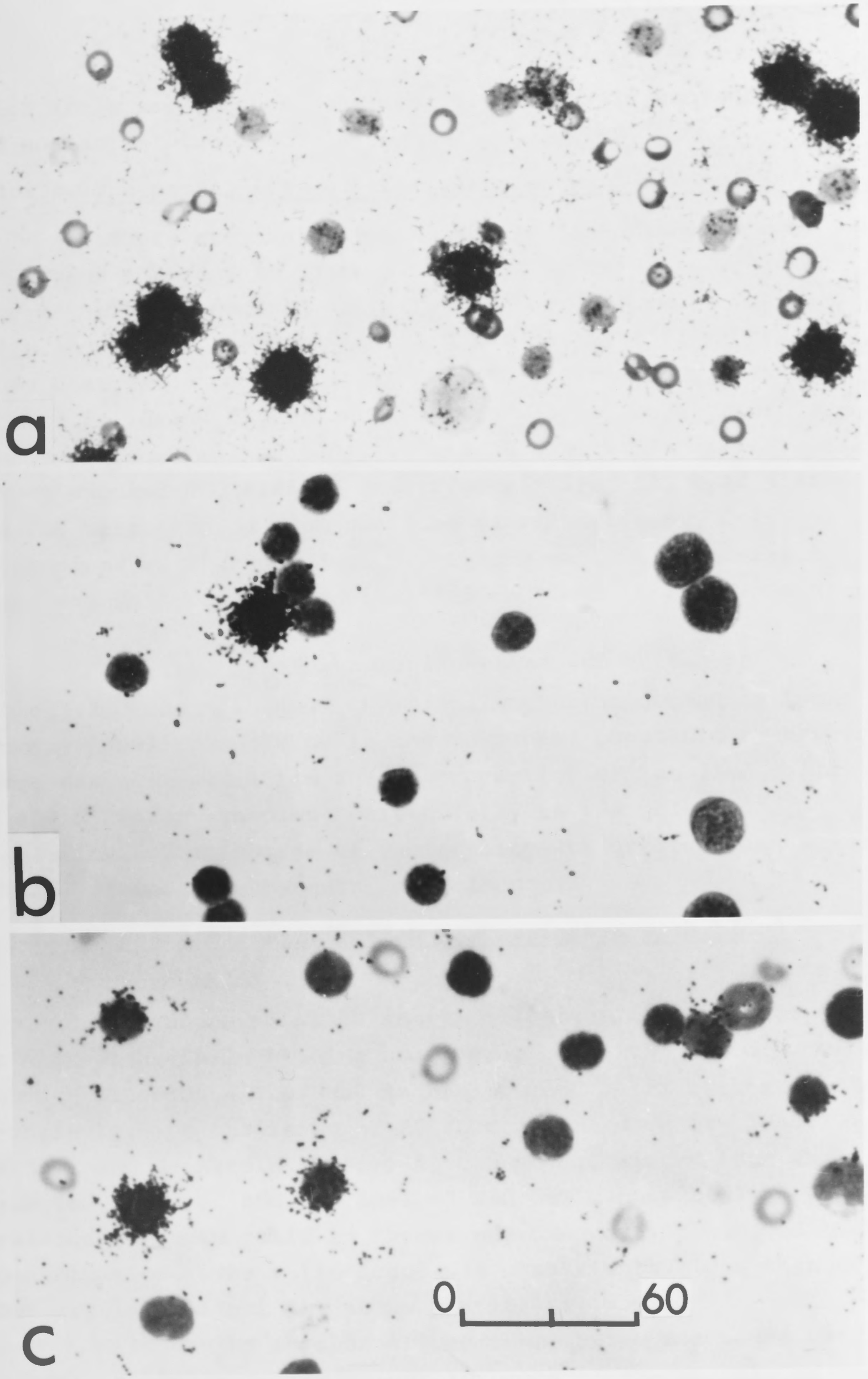
(a) spleen

(b) thymus

(c) bone marrow,

reacted under "standard" conditions, after 4 days' exposure.

Scale in microns.


a
b
c
0
60

which arose was whether these cells were merely contaminants and not an intrinsic thymic population.

2.3.3.1 The possibility of contamination with lymph node cells

There are small lymph nodes on the dorsal side of the thymus adherent to the capsule, and unless care is taken they may also be excised when thymuses are removed from mice, (Blau and Gaugas, 1968). Leckband and Boyse, (1970) have shown that these parathymic lymph node cells can be readily located following injection of carbon black. Suspensions of carbon black were injected i.p. and 30 minutes later the mouse thymus exposed and excised (see Figure 2.5). All dyed tissue and fat were removed from one lobe of each thymus. Cell suspensions were prepared as usual from each lobe and reacted under standard conditions with ^{125}I -anti-mouse light-chain Ig.

Results of radioautography are summarized in Table 2.2. The "cleaned" thymus cell population had at least as many labelled cells as in the untouched population. However, there was some variation in the proportion of labelled cells in the different samples, particularly in the "cleaned" population. This degree of variability was greater than was normally found in subsequent experiments.

2.3.3.2 Possible contamination of the thymus with blood borne cells

A second possible source of contamination of thymus is anti-light-chain binding cells present in the blood. It was found that about 40% of WBC in adult blood label with anti-CBA L-chain Ig. As the ratio of RBC:WBC in CBA mice is 1700:1 (Russel and Bernstein, 1966), it can be calculated that the expected ratio of RBC to labelled WBC would be 4250:1 if the presence of these cells in thymus was due to simple blood contamination. The ratio found was consistently less than 40:1, thus arguing against the above possibility.

It can be further argued that the thymus would need to contain 10-20% of its weight in blood if the calculated content of labelled WBC (3×10^4/thymus) were due to blood present in the organ and this is not the case. There might, of course, be preferential accumulation of WBC in the thymus.

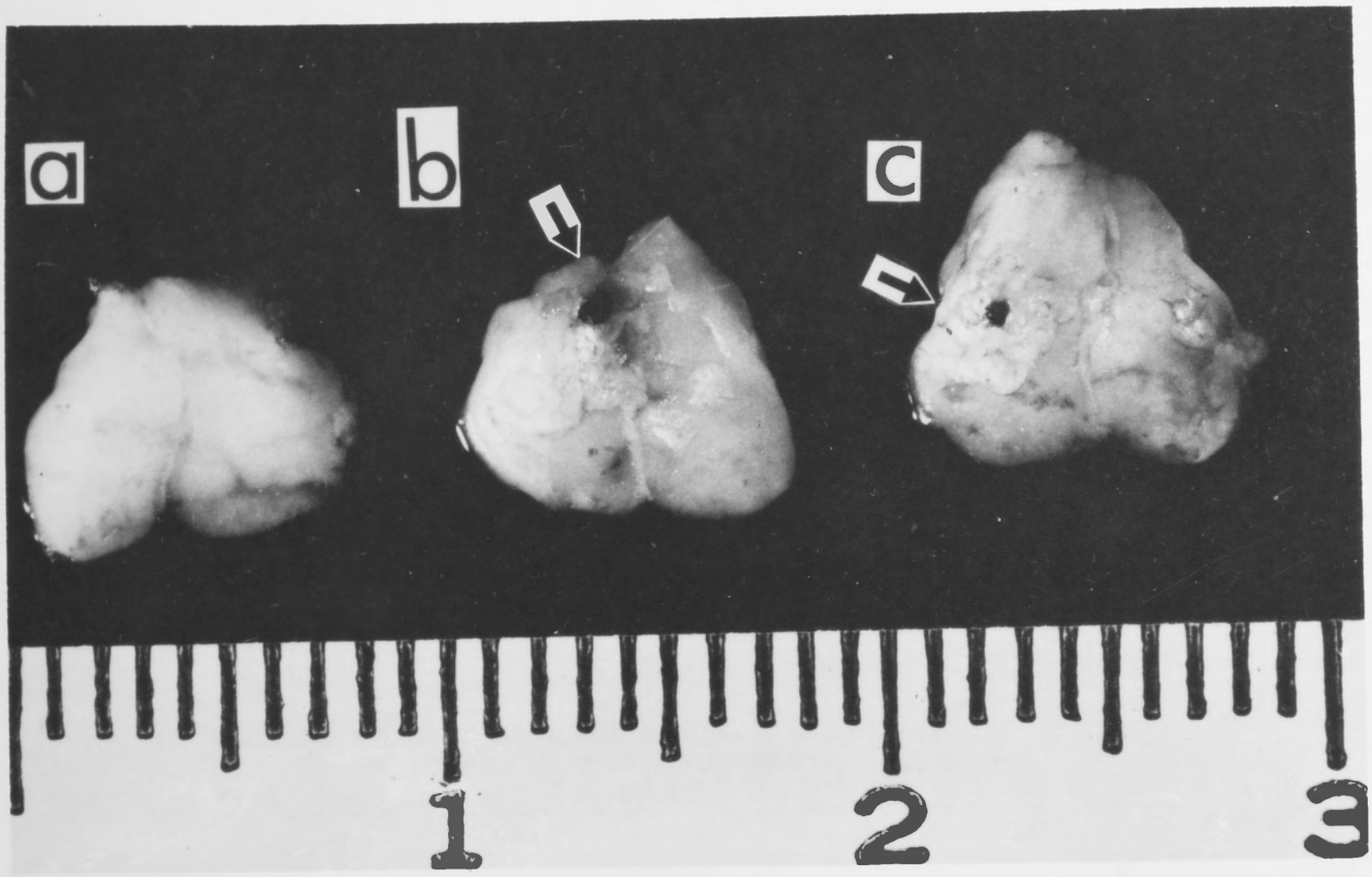


Figure 2.5 : Carbon black visualization of parathymic lymph nodes

(a) A thymus from which all surrounding dyed tissue and fat was removed - "cleaned" thymus.

(b) and (c) Thymuses showing fatty tissue and parathymic lymph nodes (arrowed) adhering to the capsule.

Table 2.2: Evidence for intrinsic nature of anti-light chain binding cells in thymus: I

Possible lymph node contamination

Thymus	Percentage of labelled cells (a) Cleaned lobe	(b) Untouched lobe
1	0.027	0.027
2	0.057	0.023
3	0.155	0.042
4	0.034	0.022

Table 2.3: Evidence for intrinsic nature of anti-light chain binding cells in thymus: II

Injection of labelled lymphocytes into mice

Time after injection	Percentage of labelled cells in smears		Average no. of cells per cross section (approx. 10^6 cells)[a]	
	Spleen	Thymus	Spleen	Thymus
10 min	0.02	<0.001	61	0
20 min	0.03	<0.001	43	0
40 min	0.06	<0.001	58	0
60 min	0.08	<0.001	94	0.2
120 min	0.07	<0.001	116	0
Overnight	<0.005	<0.001	0.3	0

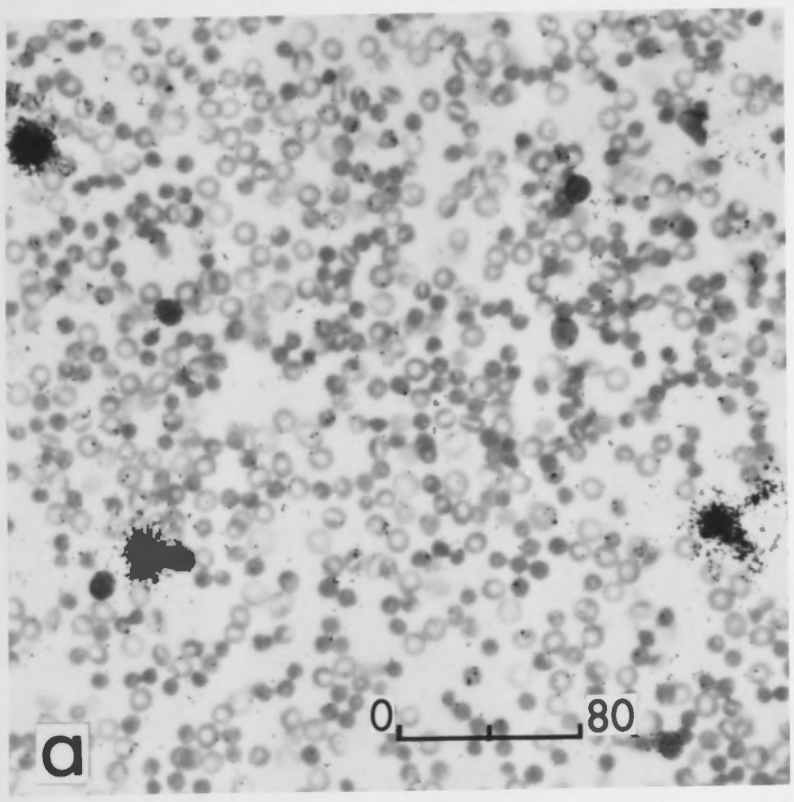
a) 5 Sections through different levels of each tissue were counted.

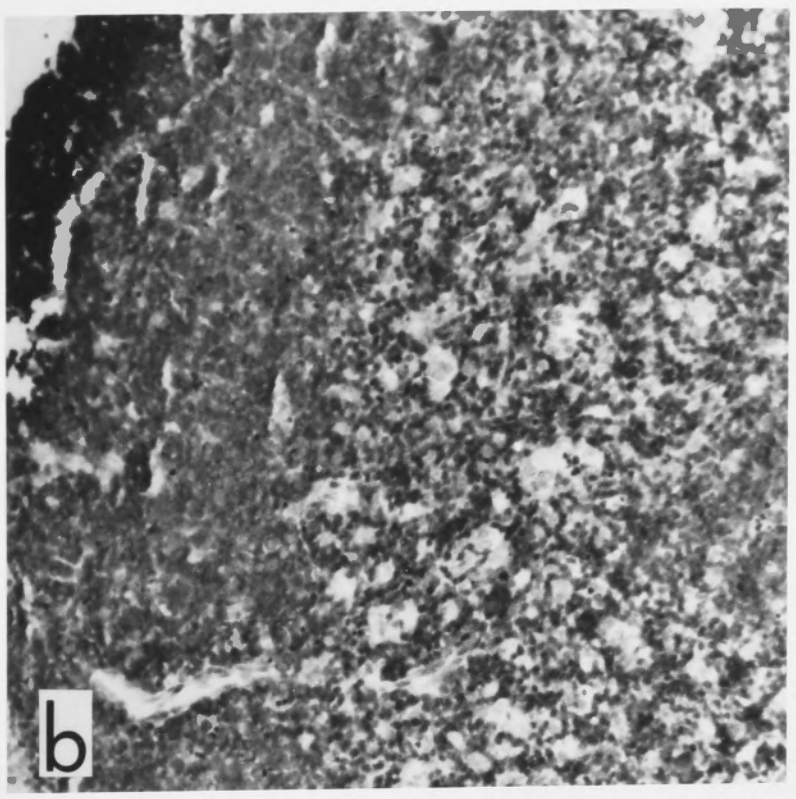
Figure 2.6

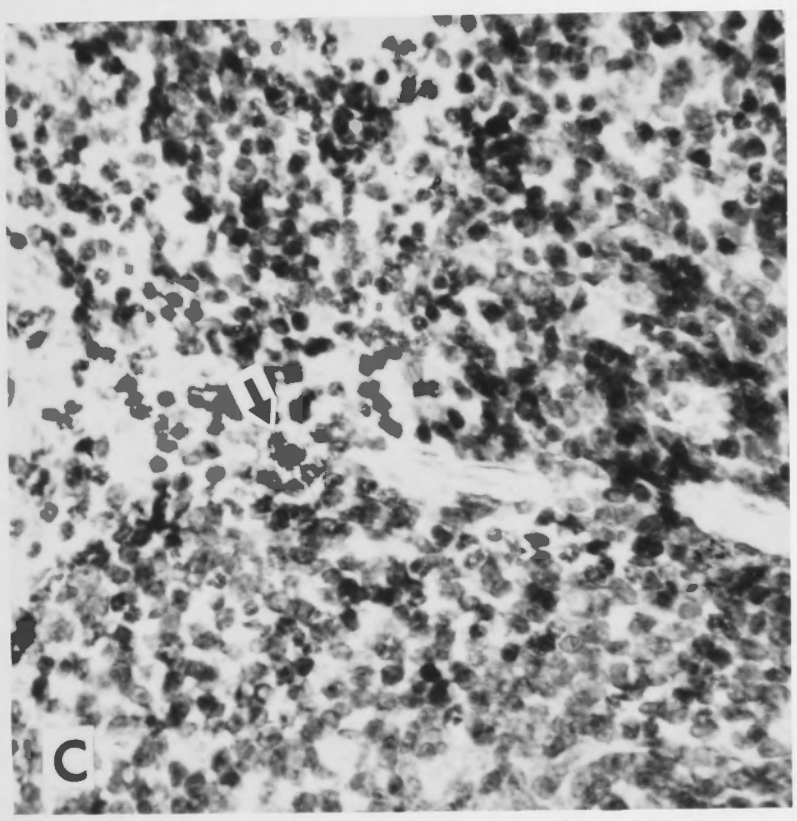
(a) Labelled WBC present in the blood of recipient mice a few minutes after their injection. Scale in microns.

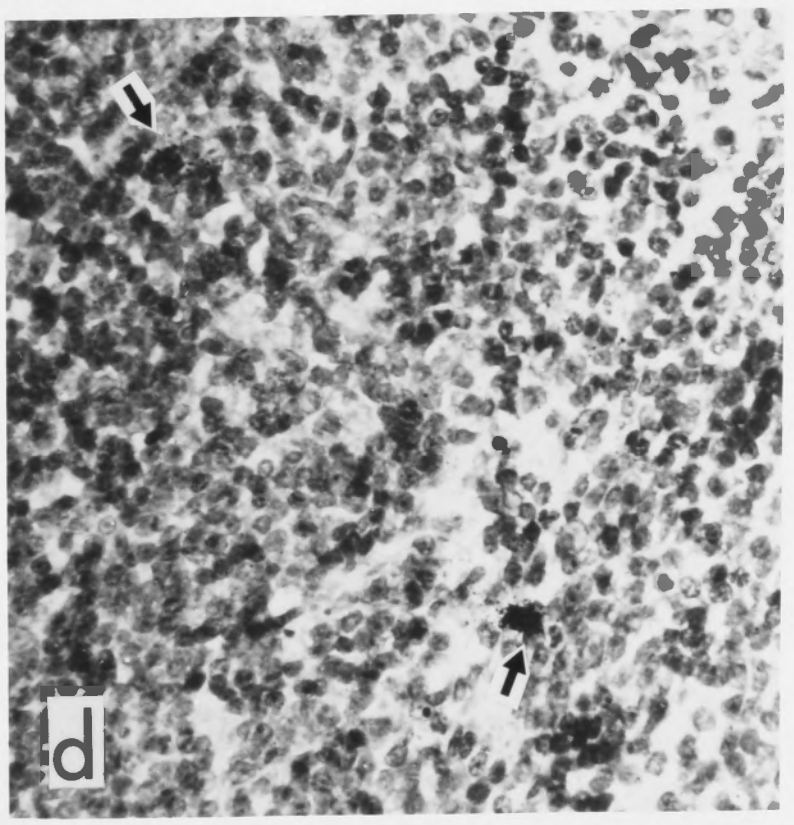
(b) A low power (approximately 40x magnification) of a typical thymus section, 10 minutes to 120 minutes after inoculation of labelled WBC. No labelled cells were apparent.

(c) and (d) Higher power view of spleen sections (approximately 200x magnification) 20 minutes and 60 minutes after inoculation of labelled WBC. Labelled cells (arrowed) were seen in blood vessels.


a
0
80


b


c


d

2.3.3.3 Injection of labelled lymphocytes into mice

A further approach to this problem was to see if WBC pre-labelled with radioactive anti-light-chain Ig and injected i.v. into mice would be recovered in the thymus.

WBC were isolated, labelled with anti-mouse light chain IgG and about 2×10^6 WBC (i.e. 8×10^5 labelled cells) in 0.2 ml were injected i.v. into recipient mice. This represents 5-10% of the WBC circulating in the mouse. At various time intervals thereafter (10 mins - 24 hours) the mice were killed and thymuses and spleens removed. One lobe of each thymus and half of each spleen were fixed in formalin and sections cut and examined by radioautography. In each case five sections through different levels of the organ were obtained. Single cell suspensions of the other portions of the organs were prepared and smears of these also subjected to radioautography. The presence (or absence) of labelled cells in the sections and smears is recorded in Table 2.3.

Labelled cells were found in both smears and sections of spleen at all times up to 2 hours. In the sections, these cells appeared to be present in the blood vessels. No unequivocally labelled cells were found in either smears or sections of the thymus (Figure 2.6). Thus there was again no evidence to suggest that the anti-light chain binding cells in the thymus were due to simple blood contamination.

2.3.4 Reproducibility

In many separate experiments in which the reaction of thymocytes with ^{125}I -labelled anti-mouse light chain was studied under comparable conditions ("standard conditions") approximately the same percentage of labelled cells was consistently observed, the mean value being 0.036% ± 0.005, range : 0.02 - 0.1%.

2.3.5 Proportion of anti-light chain binding cells in thymuses of different mouse strains

The thymuses of mice of different strains were found to contain similar proportions of cells binding anti-L chain IgG (Table 2.4).

Table 2.4: Incidence of anti-light chain binding cells in thymuses of different mouse strains

Strain a)	Percentage of labelled cells b)
CBA/J	0.01, 0.02
CBA/H	0.03, 0.02
C57/BL 6J	0.05, 0.02
(C57/BL X CBA/J)F_1	0.05, 0.04
BALB c	0.09, 0.08

a) In all cases mice were 7-9 weeks of age.

b) Duplicate samples.

2.3.6 Reaction of thymocytes with ^{125}I -Fab of anti-light chain Ig

The same proportion of labelled cells (approx. 0.03%) was also found when the Fab portion of the anti-light chain IgG (papain digest) (Porter, 1959) was labelled and bound to CBA thymocytes under standard conditions. This binding was also inhibited by pre-treatment with cold anti-mouse light chain Ig but not by pre-treatment with cold anti-sheep light chain Ig. (Figure 2.7.)

2.3.7 The dose dependence of anti-light chain binding cells in different tissues

Spleen, bone marrow and thymus cell suspensions were incubated with concentrations of ^{125}I -anti-light chain Ig varying from 43-2200 ng per 2×10^7 cells, and radioautography carried out as usual (Table 2.5).

The results indicated clearly that whereas the number of binding cells found in spleen and bone marrow was only slightly dose dependent, there was a marked effect with thymus, the numbers of labelling cells increasing up to 50 times as the concentration of the reagent increased 50 fold; i.e. 2-5 times the number obtained under standard conditions.

A further substantial difference between spleen and bone marrow, on the one hand, and thymus on the other was shown if the frequency of grains over cells seen with the highest dose of anti-light chain was plotted (Figure 2.8). The majority of cells in both spleen and bone marrow were heavily labelled whereas the majority of cells in thymus were lightly labelled, thus altering the pattern seen with the "standard" dose of anti-light chain (Figure 2.1).

2.3.8 The age incidence of anti-light chain binding cells in different tissues

The proportion of anti-light chain binding cells found in the bone marrow, spleen, blood and thymus of mice of varying ages - 6-7 weeks, 3 weeks, 1 week and 3-4 days - are summarized in Table 2.6 and Table 2.7. In the case of spleen and blood, and particularly with bone marrow, there was a sharp drop in the number of anti-light chain binding cells in very young mice compared to older mice. Results for the thymus

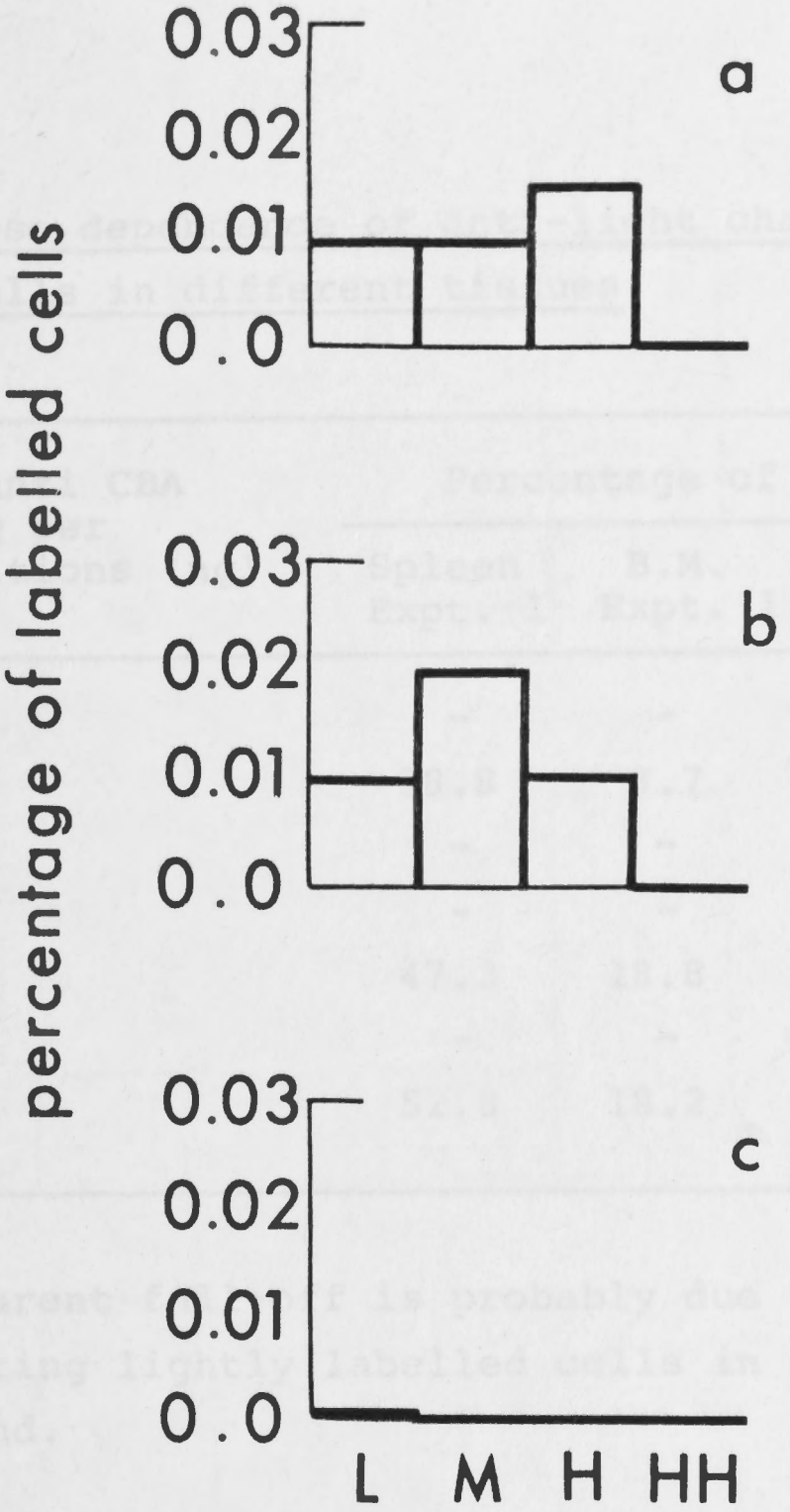


Figure 2.7 Reaction of thymocytes with ^{125}I-Fab of anti-light chain 1g.

(a) The proportion of labelled cells which reacted with the Fab reagent (0.04%).

(b) The proportion of labelled cells following pre-treatment with cold anti-sheep light chain (0.04%).

(c) The proportion of labelled cells found following pre-treatment with cold anti-mouse light chain (0.0008%).

'L' : < 25 grains; 'M' : 25-50 grains; 'H' : > 50 grains; 'HH' : "hedgehog" - cell obscured by density of grains.

Table 2.5: Dose dependence of anti-light chain binding cells in different tissues

Dose of ^{125}I-anti CBA light chain Ig per standard conditions (ng)	Percentage of cells labelled			
	Spleen Expt. 1	B.M. Expt. 1	Thymus Expt. 1	Thymus Expt. 2
43	-	-	-	0.01
80	38.8	8.7	0.047	0.05
220	-	-	0.08	0.12
430	-	-	-	0.27
700	47.3	18.8	-	-
860	-	-	-	0.49
2200	52.8	18.2	0.43	0.26 a)

a) This apparent fall-off is probably due to the difficulty in detecting lightly labelled cells in slides with high background.

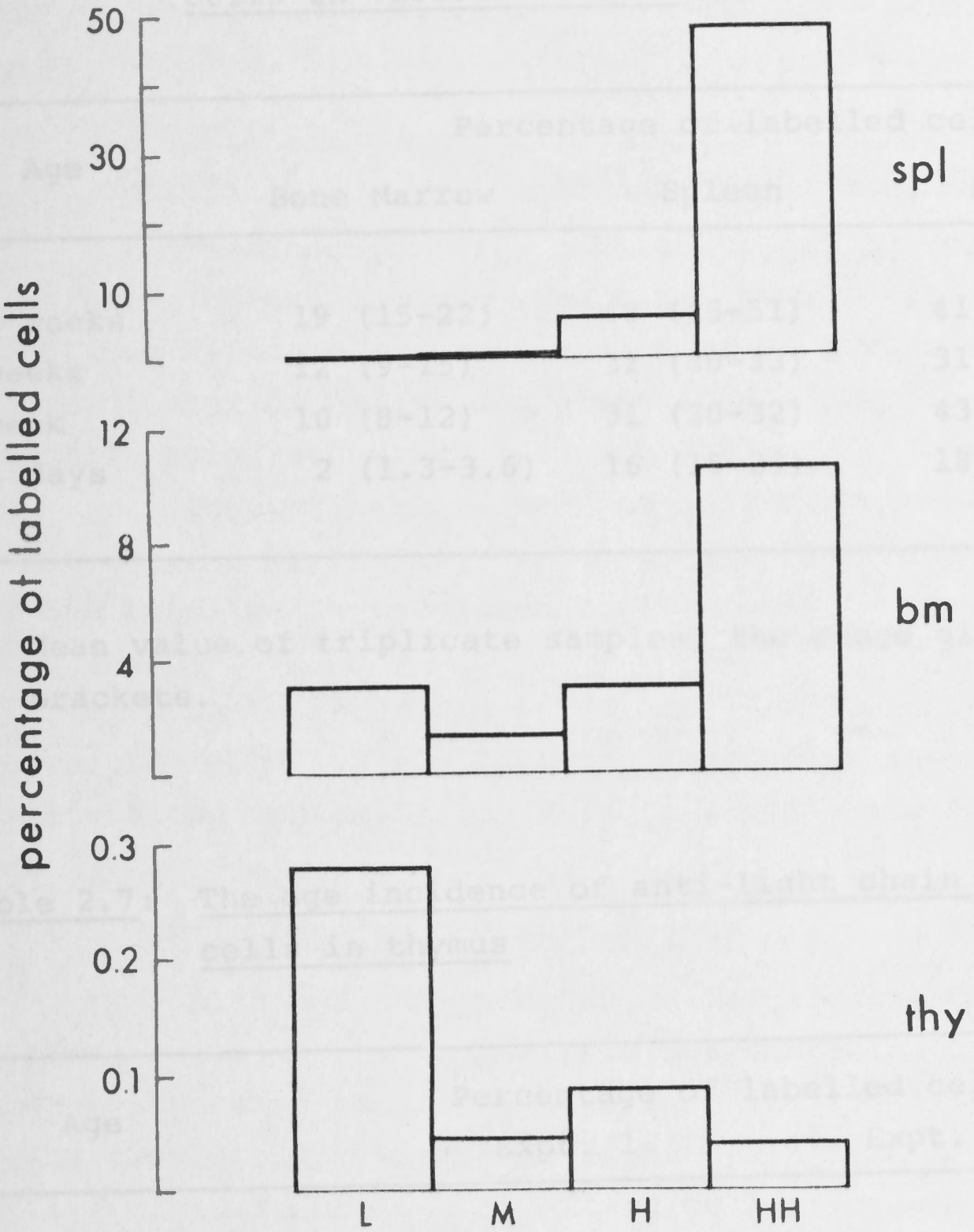


Figure 2.8 Frequency of grains over cells from different tissues seen with the highest dose of anti-light chain lg (2200ng).

'L' : < 25 grains; 'M' : 25-50 grains; 'H' : > 50 grains; 'HH' : "hedgehog" - cell obscured by density of grains.

Table 2.6: The age incidence of anti-light chain binding cells in various tissues

Age	Percentage of labelled cells a)		
	Bone Marrow	Spleen	Blood
6-7 weeks	19 (15-22)	48 (45-51)	41 (39-42)
3 weeks	12 (9-15)	32 (30-33)	31 (29-32)
1 week	10 (8-12)	31 (30-32)	43 (40-43)
3-4 days	2 (1.3-3.6)	16 (15-20)	18 (14-21)

a) Mean value of triplicate samples, the range given in brackets.

Table 2.7: The age incidence of anti-light chain binding cells in thymus

Age	Percentage of labelled cells	
	Expt. 1	Expt. 2
6-7 weeks	0.01, -	0.02
3 weeks	0.01, 0.04	0.05
1 week	0.04, 0.03	0.03
3-4 days	0.02, 0.04	0.01

a) Duplicate smears.

are variable, in part due to the small numbers of labelled cells involved, but no such trend is apparent.

2.3.9 The effect of cortisone treatment on the proportion of anti-light chain binding cells in thymus

Treatment of mice with cortisone causes rapid involution of the thymus. The total numbers of cells recovered 24 hours after injection of cortisone was 3% of the normal number of cells, and after longer periods as few as 1% of cells were recovered (Figure 2.9).

However, the numbers of anti-L chain binding cells were enriched by this cortisone treatment i.e. as a fraction of the total cells recovered their numbers increased about 10 fold (Table 2.8).

Again the ratio of RBC to labelled cell on the radioautographs was 10-100 times lower than expected if simple blood contamination were to explain the increase.

2.3.10 The effect of various antisera on the anti-light chain binding cells in thymus

Although the evidence so far indicated that the binding cells in the thymus were not contaminating B cells, more evidence of their thymic origin was required. Therefore cells pre-labelled with anti-light chain were treated with anti-θ globulin or with an anti-B cell serum and complement. The cortisone resistant cell population was mainly studied because of the greater frequency of binding cells. Cells lysed by the antisera were detected on radioautographs by a nigrosin staining procedure.

2.3.10.1 Anti-θ globulin

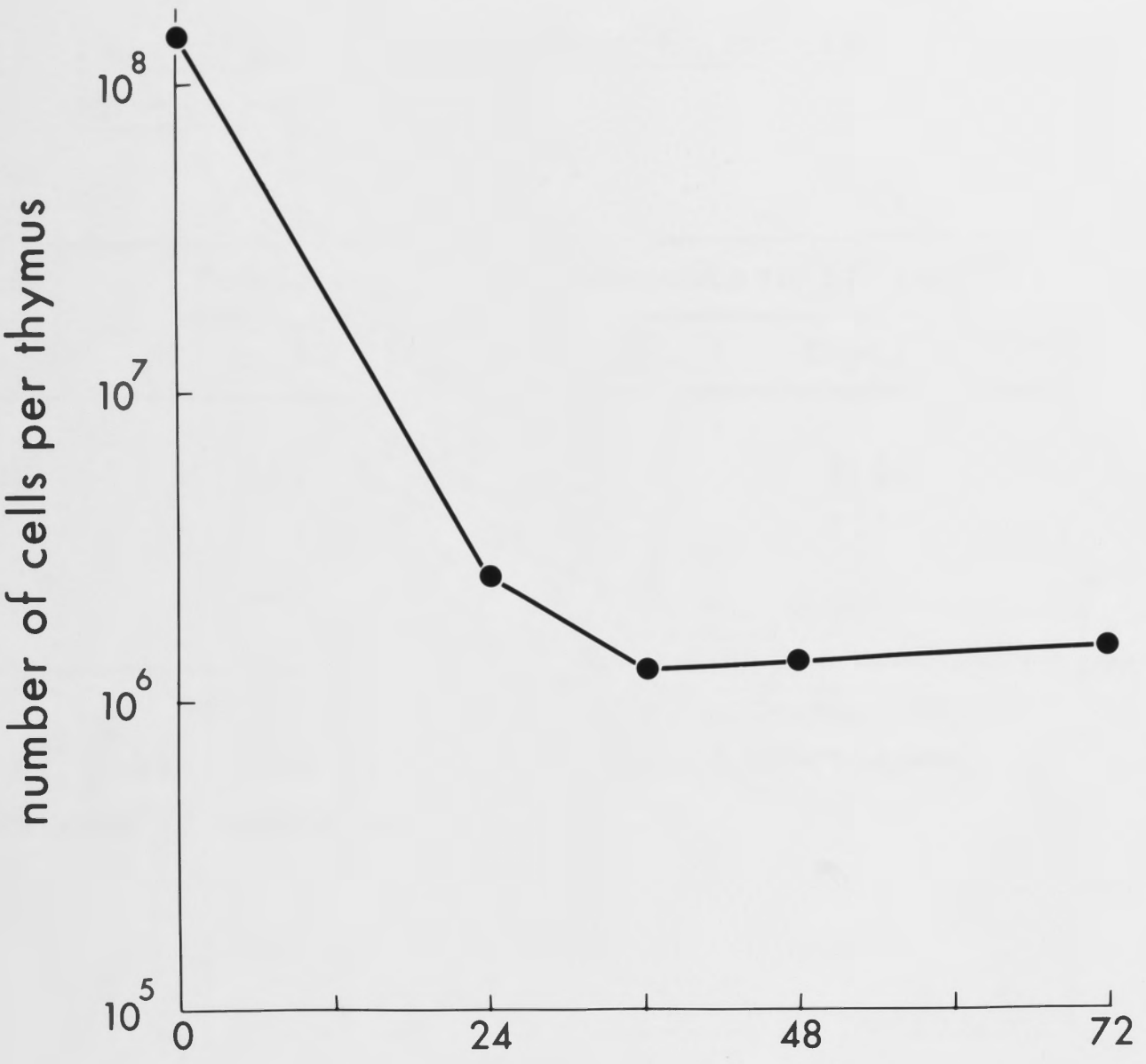
The effect of anti-θ on thymocytes and the cortisone resistant thymocytes is shown in Figure 2.10. About 1% of thymus cells were found to be resistant to anti-θ, and in the cortisone resistant fraction this proportion increased to about 10% of the cell population. Radioautographs of labelled (^{125}I -anti-light chain Ig), and stained, nigrosin cell smears indicated that although about 20% of the cells were lysed the majority of labelled cells were resistant to the anti-θ treatment under these conditions. Most of the labelled cells in

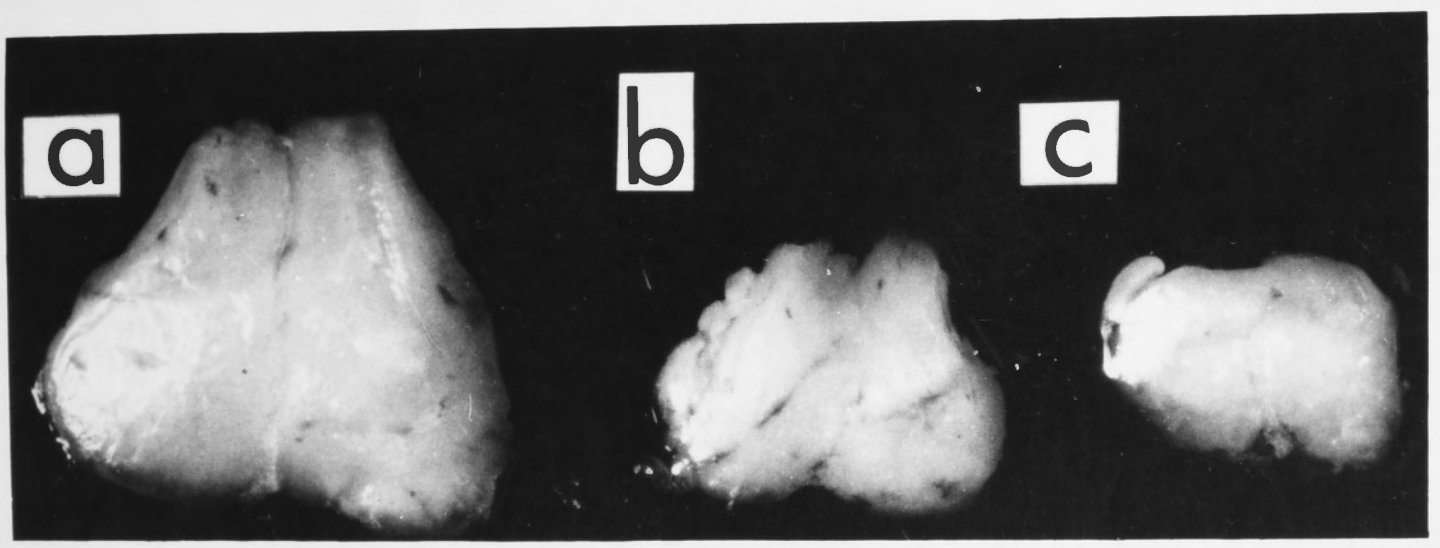
Figure 2.9

Involution of the thymus following treatment of mice with cortisyl acetate.

Upper: the numbers of viable lymphocyte like cells recovered from the thymus at different time intervals after treatment (each point is the arithmetic mean of 3 values).

Lower: the macroscopic appearance of the thymus at a) 0, b) 12 and c) 24 hours after cortisone treatment.


number of cells per thymus
10^8
10^7
10^6
10^5
0
24
48
72
time after cortisone treatment
(hours)


a
b
c


1
2

Table 2.8: Anti-light chain binding to cortisone resistant thymus cells

Time after Cortisone Treatment	Total cells recovered ($\times 10^{-6}$)	Percentage of labelled cells		
		Expt. 1	Expt. 2	Expt. 3
Untreated	150	0.02	0.03	0.03
24 hours	4	0.3	0.3	0.4
>36 hours [a]	1.5	0.2	-	-

a) Pool of cells from mice treated with cortisone 36, 48 and 72 hours previously.

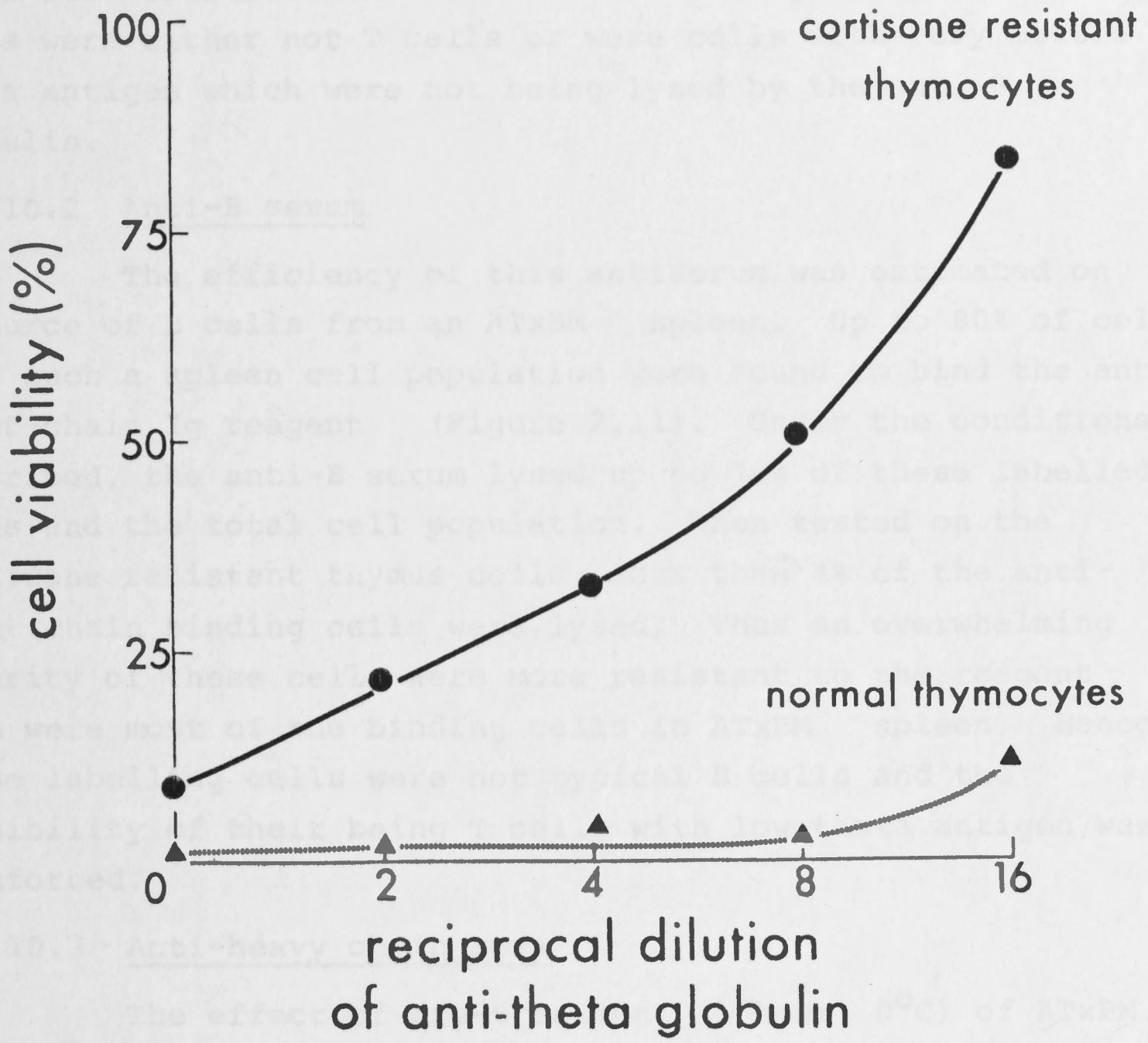


Figure 2.10 The cytotoxicity of anti-θ globulin for normal thymocytes (dotted line) and cortisone resistant thymocytes (solid line) under conditions described in the text.

the susceptible population were of the lightly labelled, larger category, described previously. It is important to point out that there were many (up to 10%) unlabelled cells which were also resistant to the anti-θ reagent. Hence these cells were either not T cells or were cells with very little theta antigen which were not being lysed by the anti-θ globulin.

2.3.10.2 Anti-B serum

The efficiency of this antiserum was estimated on a source of B cells from an ATxBM spleen. Up to 80% of cells from such a spleen cell population were found to bind the anti-light-chain Ig reagent (Figure 2.11). Under the conditions described, the anti-B serum lysed up to 70% of these labelled cells and the total cell population. When tested on the cortisone resistant thymus cells, less than 4% of the anti-light chain binding cells were lysed. Thus an overwhelming majority of these cells were more resistant to the reagent than were most of the binding cells in ATxBM spleen. Hence, these labelling cells were not typical B cells and the possibility of their being T cells with low theta antigen was reinforced.

2.3.10.3 Anti-heavy chain sera

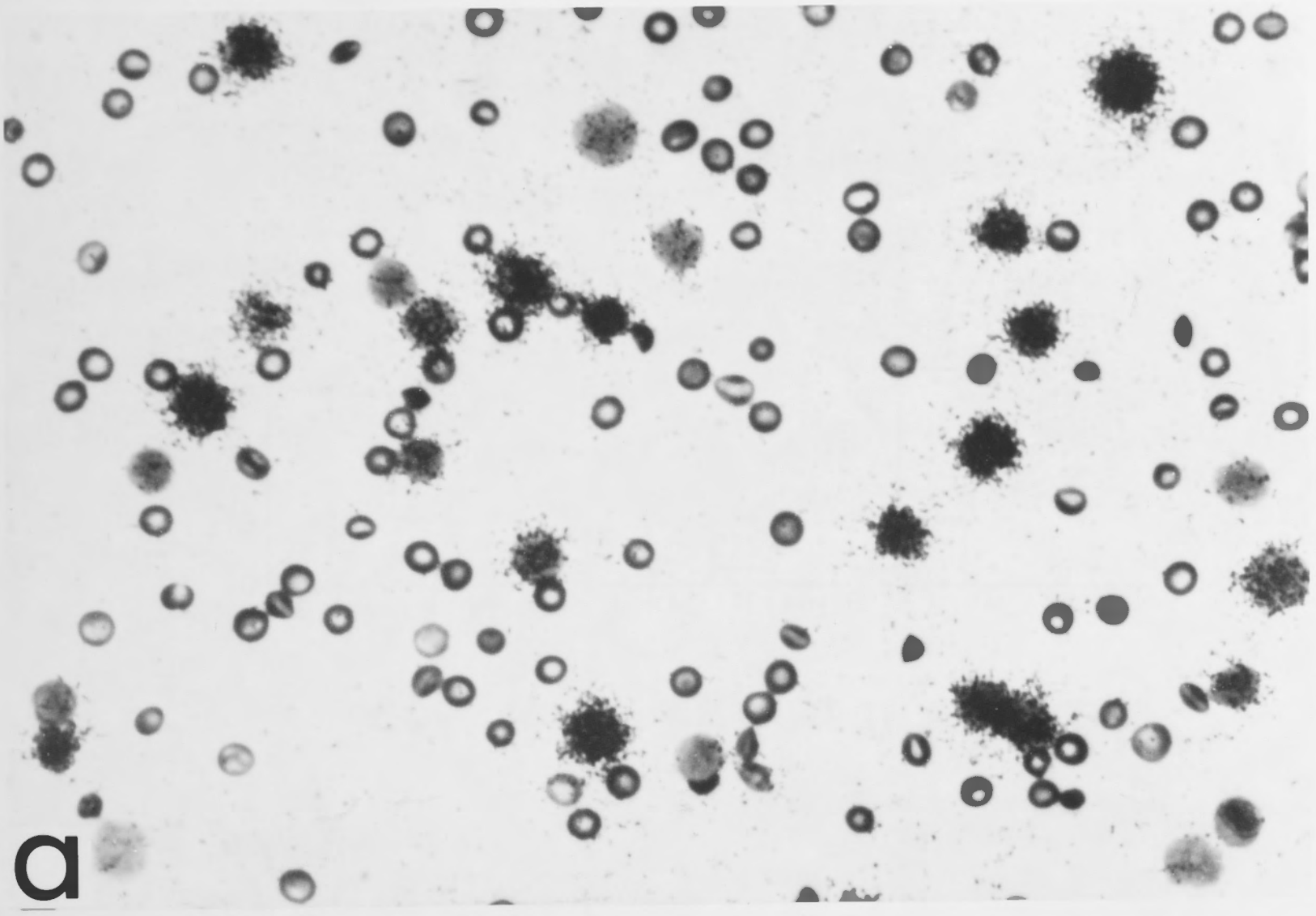
The effect of pre-treatment (1 hour, $0^{o}C$) of ATxBM spleen cells and cortisone resistant thymocytes with specific anti-μ chain and anti-γ chain sera on the anti-light chain binding cells was investigated. Both antisera inhibited the binding in both cell populations, decreasing the overall number of labelled cells and the density of grains over the remaining labelled cells. The anti-γ chain serum was 3x more effective than the anti-μ chain serum in blocking the anti-light chain binding (Figure 2.12). Three additional experiments showed that anti-μ pretreatment of cortisone resistant thymocytes decreased the total proportion of labelled cells from 0.48 to 0.39%.

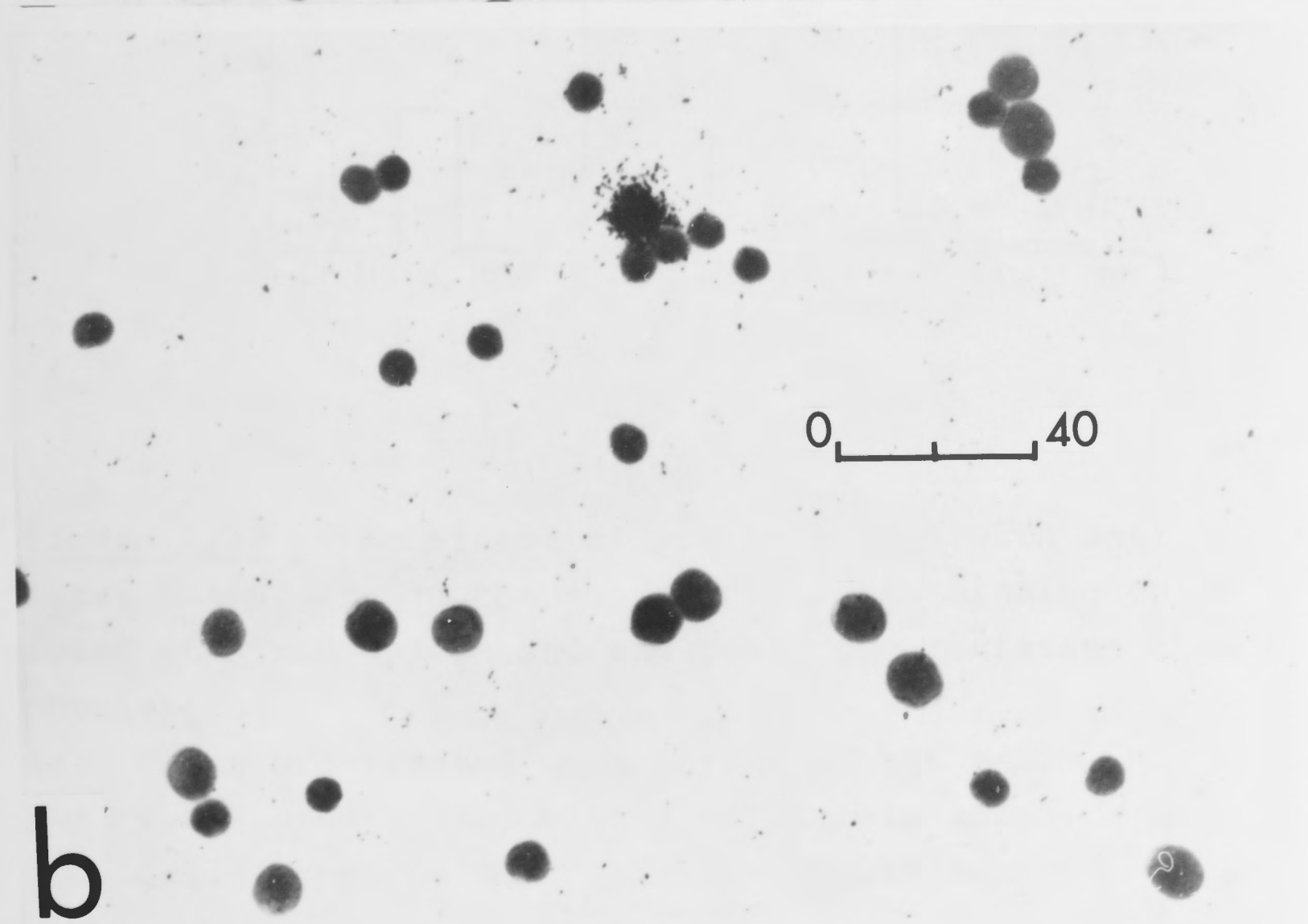
2.3.11 Estimation of total immunoglobulin present in thymocytes

The possibility remained that many more thymus cells

Figure 2.11

Comparison of anti-light-chain binding cells in
(a) ATxBM spleen and
(b) normal thymus.
("Standard" conditions, 3 days' exposure.)
Up to 80% of cells from ATxBM spleen were labelled.


a


0
40
b

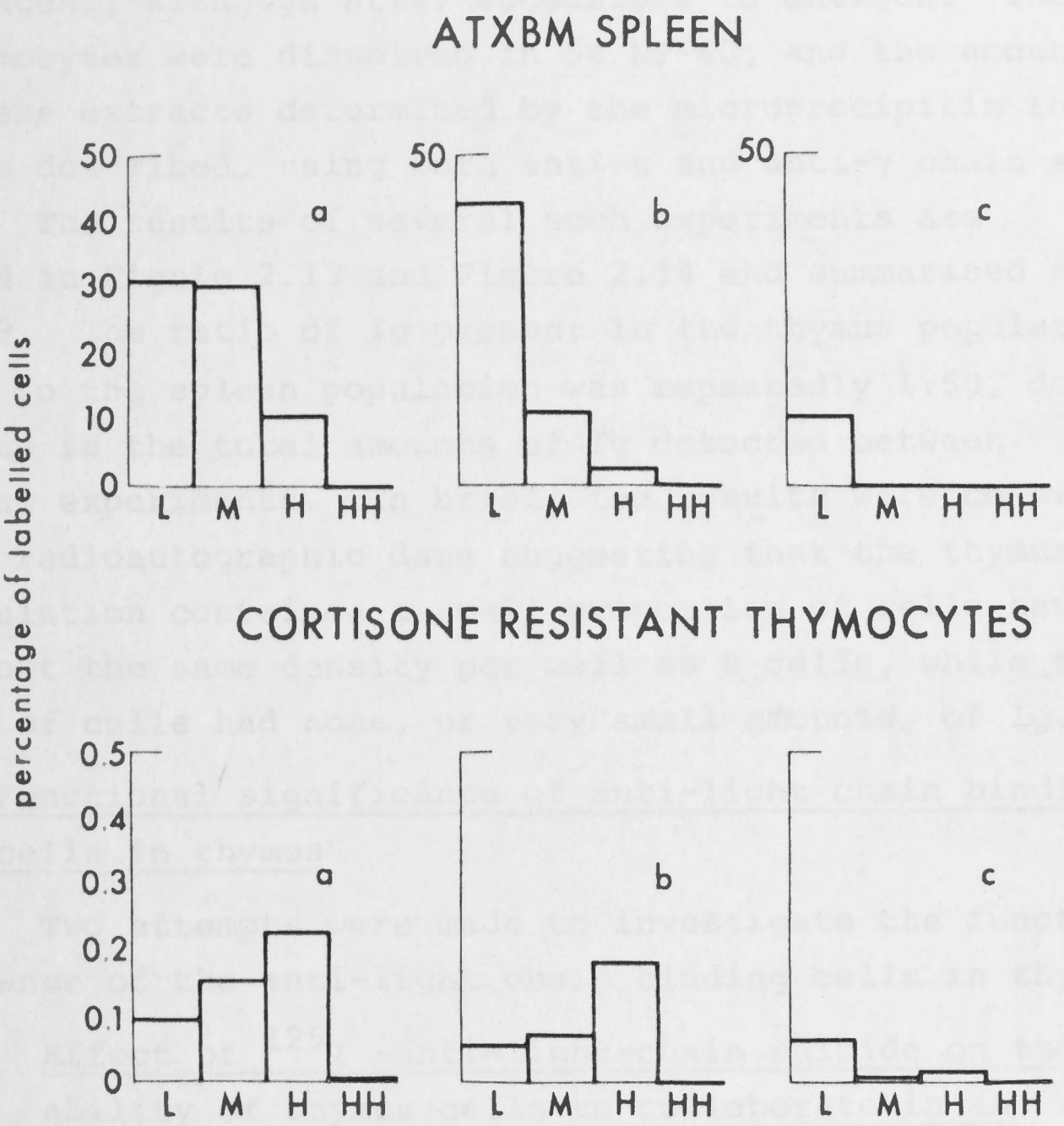


Figure 2.12 The effect of pre-treatment with anti-heavy chain sera on the anti-light chain binding cells found in ATxBM spleen and the cortisone resistant thymus population.

(a) Cells pre-treated with normal rabbit serum.

(b) Cells pre-treated with anti-μ chain serum.

(c) Cells pre-treated with anti-γ chain serum.

detected by the above procedure, contained significant amounts of Ig, possibly held in the plasma membrane in such a manner that the appropriate groups were not accessible to the anti-L-chain reagent, although still accessible to antigen. Therefore thymocytes were dissolved in 5% NP-40, and the amount of Ig in these extracts determined by the microprecipitin technique, as described, using both anti-μ and anti-γ chain sera.

The results of several such experiments are presented in Figure 2.13 and Figure 2.14 and summarised in Table 2.9. The ratio of Ig present in the thymus population compared to the spleen population was repeatedly 1:50, despite variations in the total amounts of Ig detected between individual experiments. In brief, the results were consistent with the radioautographic data suggesting that the thymus cell population contained a small proportion of cells having Ig at about the same density per cell as B cells, while the majority of cells had none, or very small amounts, of Ig.

2.3.12 Functional significance of anti-light chain binding cells in thymus

Two attempts were made to investigate the functional significance of the anti-light chain binding cells in thymus.

2.3.12.1 Effect of ^{125}I -anti-light-chain suicide on the ability of thymus cells to collaborate in an anti-SRBC response in vivo

Spleen, bone marrow and thymus cells were reacted with ^{125}I-labelled anti-light chain reagent of high specific activity as described by Cooper and Ada (1972). The cells were then injected into syngeneic irradiated (850 rads) recipients. Mice were challenged 2 days later with 2×10^8 SRBC and given a secondary challenge on day 5. On day 8 spleens were harvested and assayed for anti-SRBC PFC.

Results of two such experiments are summarized in Table 2.10. While there was a marked decrease in the response when spleen and bone marrow cells were suicided, thymus activity was completely unaffected under the experimental conditions used.

2.3.12.2 The effect of removal of Ig positive rosette forming cells on the ability of thymus cells to collaborate in an anti-hapten response in vitro

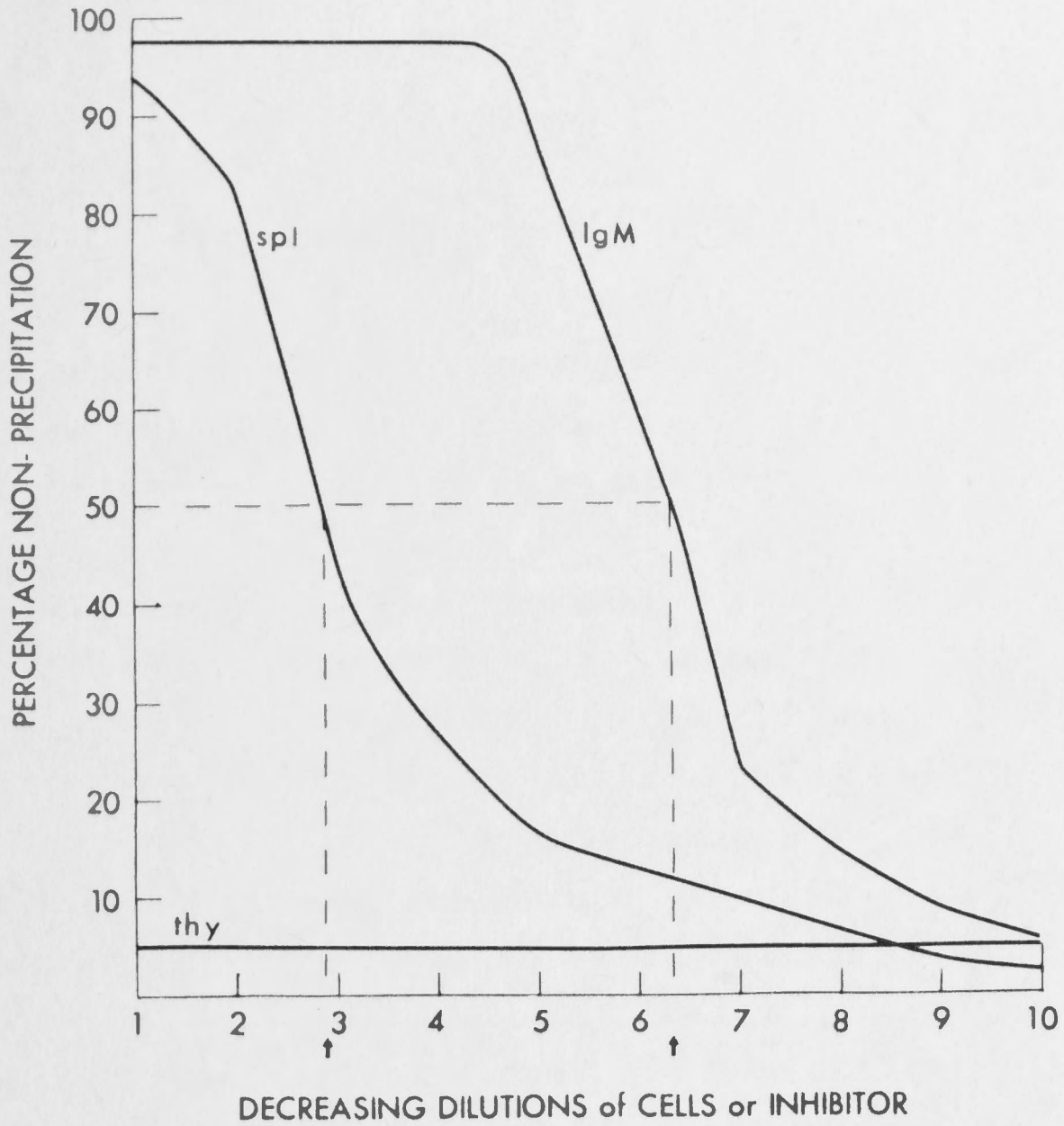


Figure 2.13

Microprecipitin inhibition curves for spleen and thymus NP-40 cell extracts. (Experiment 1).

The total amount of Ig present in spleen and thymus was in this experiment measured by the ability of cell extracts and an IgM standard to interfere with the precipitation of ^{125}I-IgM by anti-light chain serum.

Dilution 1 is equivalent to the extract from 5.6×10^6 cells (spleen and thymus) and 177 ng IgM standard.

The 50% end-points (arrowed) were:

(i) IgM standard: 4ng

(ii) Spleen: equivalent to the extract from 1.5×10^6 cells.

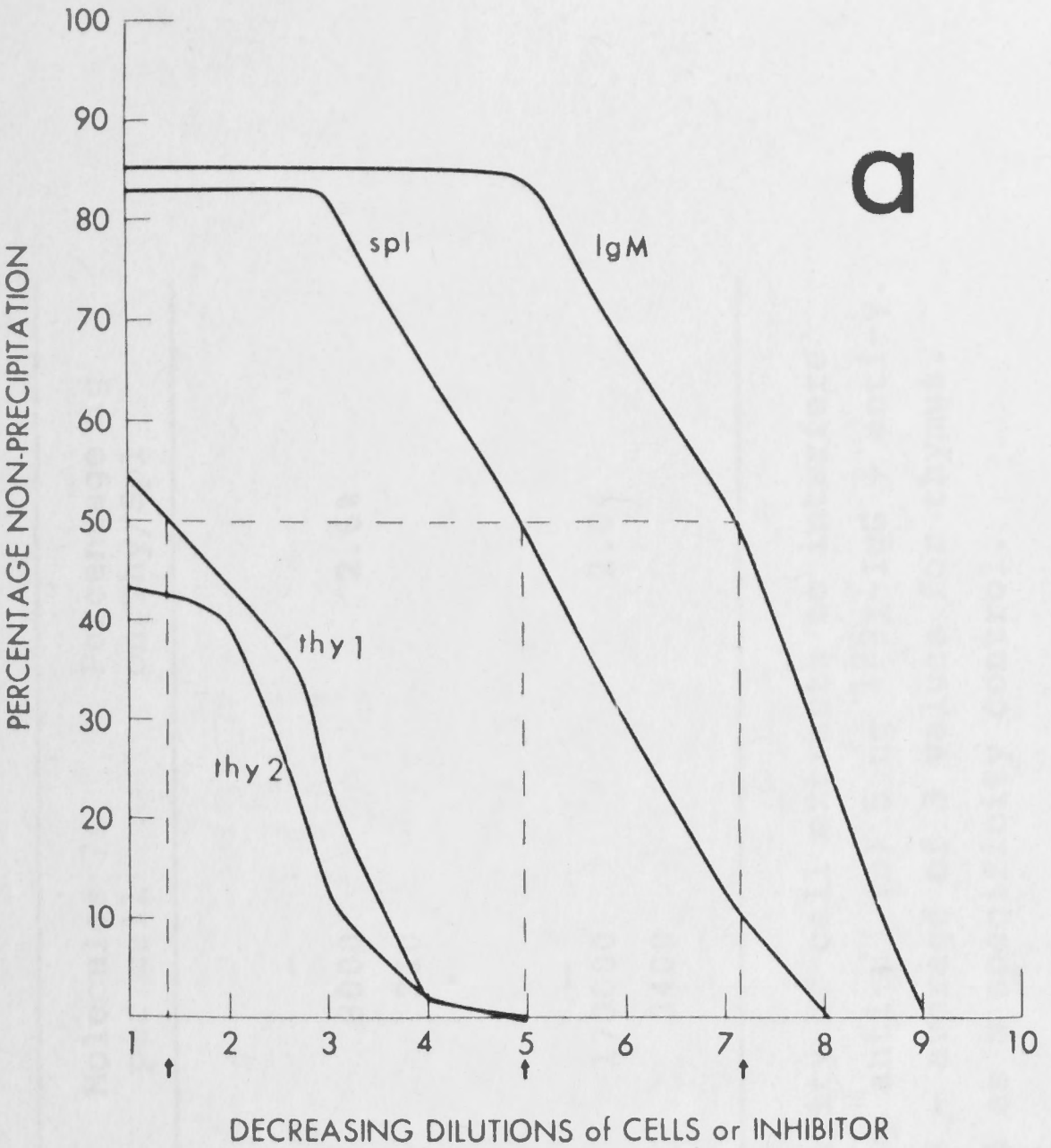
(iii) Thymus: well below the level of detection.

Figure 2.14

Microprecipitin inhibition curves for spleen and thymus NP-40 cell extracts (Experiment 2).

(a) The amount of IgM present in cell extracts was quantitated by its ability, relative to a known IgM standard, to interfere with the precipitation of ^{125}I-IgM (5ng) by specific anti-μ chain serum.
Dilution 1 is equivalent to the extract from 3.1×10^7 spleen cells, 8.2×10^7 thymus 1 cells, 7.7×10^7 thymus 2 cells and 275 ng IgM standard.
The 50% end-points (arrowed) are:
(i) IgM standard: 4ng.
(ii) Spleen: equivalent to extract from 2×10^6 cells.
(iii) Thymus 1: equivalent to extract from 8.2×10^7 cells.
(iv) Thymus 2: just below the level of detection.

(b) The amount of IgG present in cell extracts was quantitated by its ability, relative to a known IgG standard to interfere with the precipitation of ^{125}I-IgG (5ng) by specific anti-γ chain serum.
Dilution 1 is equivalent to the extract from 3.1×10^7 spleen cells, 8.2×10^7 thymus 1 cells, 7.7×10^7 thymus 2 cells and 275 ng IgG standard.
The 50% end points (arrowed) are:
(i) IgG standard : 17ng.
(ii) Spleen : equivalent to extract from 4×10^5 cells.
(iii) Thymus 1 : equivalent to extract from 2×10^7 cells.
(iv) Thymus 2 : equivalent to extract from 2.2×10^7 cells.


a
PERCENTAGE NON-PRECIPITATION
100
90
80
70
60
50
40
30
20
10
spl
IgM
thy 1
thy 2
1
2
3
4
5
6
7
8
9
10
DECREASING DILUTIONS of CELLS or INHIBITOR

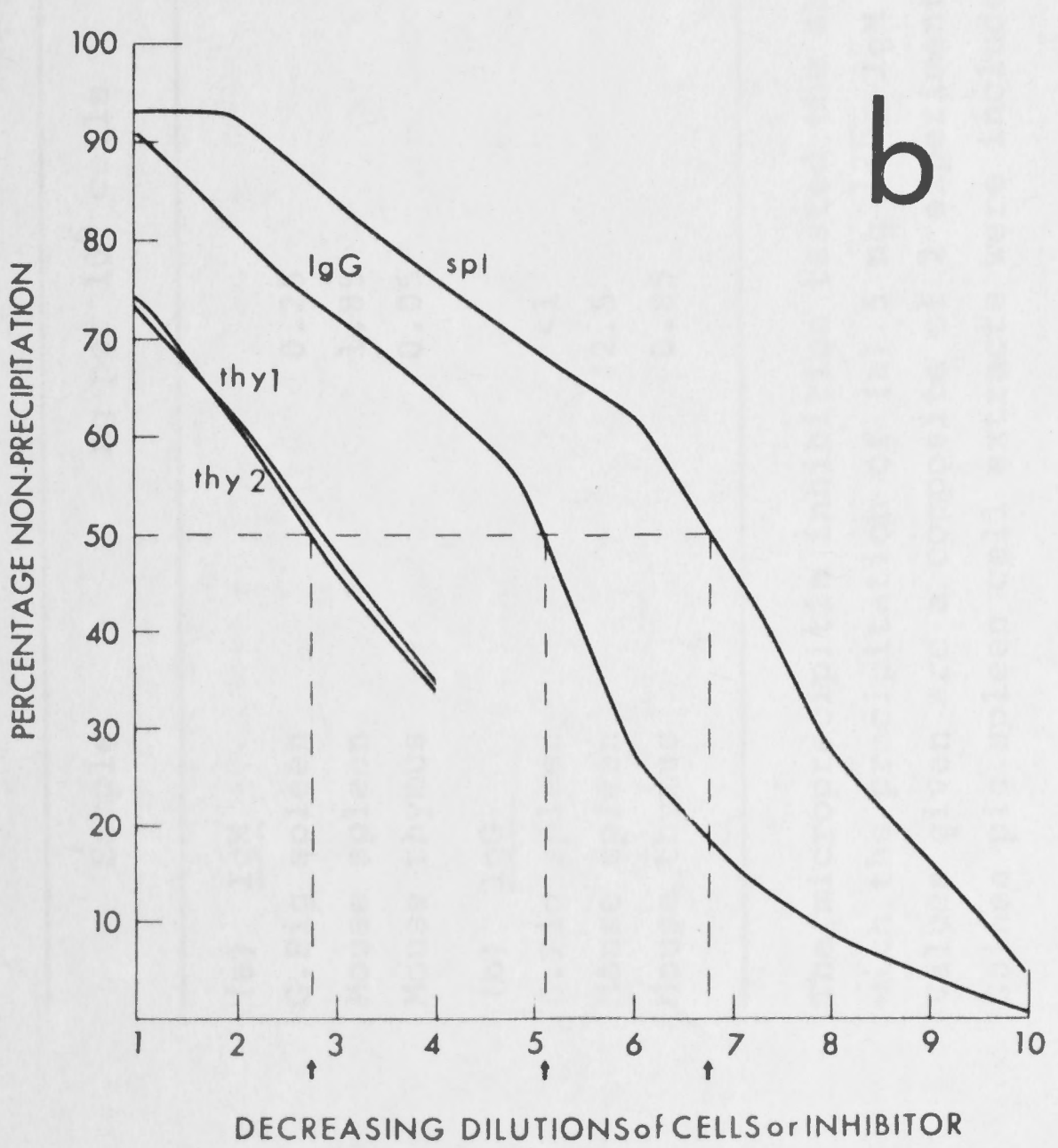

b
PERCENTAGE NON-PRECIPITATION
100
90
80
70
60
50
40
30
20
10
IgG
spl
thy1
thy 2
1
2
3
4
5
6
7
8
9
10
DECREASING DILUTIONS of CELLS or INHIBITOR

Table 2.9: Estimation of immunoglobulin in spleen and thymus NP-40 extracts (Microprecipitin inhibition)

Sample	ng per 10^6 cells	Molecules 7s per cell	Percentage Ig on Thy/Spl
(a) IgM			
G.Pig spleen	0.25	-	
Mouse spleen	1.85	8000	2.6%
Mouse thymus	0.05	210	
(b) IgG			
G.Pig spleen	<1	-	
Mouse spleen	42.5	170000	2.0%
Mouse thymus	0.85	3400	

The microprecipitin inhibition tested the ability of cell extracts to interfere with the precipitation of (a) 5 ng ^{125}I-IgM by anti-μ (b) 5 ng ^{125}I-IgG + anti-γ. Values given are a composite of 2 experiments - average of 3 values for thymus. Guinea pig spleen cell extracts were included as a specificity control.

Table 2.10: The effect of suicide with ^{125}I- anti-light chain reagent of high specific activity on the anti-SRBC response in vivo.

Cells transferred a)	Anti-SRBC PFC per spleen c) Expt. 1	Expt. 2
^{127}I treated spleen b)	4820 ± 720	n.d.
^{125}I treated spleen b)	30 ± 17	n.d.
^{127}I treated bone marrow	n.d. d)	186 ± 70
^{125}I treated bone marrow	n.d.	162 ± 28
^{127}I treated thymus	n.d.	160 ± 20
^{125}I treated thymus	n.d.	24 ± 14
^{127}I treated bone marrow + ^{127}I treated thymus	1480 ± 420	1664 ± 420
^{125}I treated bone marrow + ^{127}I treated thymus	760 ± 100	96 ± 20
^{127}I treated bone marrow + ^{125}I treated thymus	1940 ± 660	1106 ± 480
^{125}I treated bone marrow + ^{125}I treated thymus	800 ± 97	166 ± 40
No cells	20 ± 8	60 ± 20

a) In experiment 1, approximately 2×10^7 spleen, 1.3×10^7 bone marrow and 8.5×10^7 thymus cells were transferred i.v. in 0.3 ml of medium into syngeneic mice which had been lethally irradiated (850 rads) 24 hours previously.
In experiment 2, approximately 1.9×10^7 bone marrow and 1.2×10^8 thymus cells were transferred.

b) Cells were incubated (0°C, 30 min) at 5×10^7 - 1×10^8 per ml in medium containing 15 mM sodium azide and 5-10 µg/ml of iodinated anti-light chain reagent. ^{127}I is the non-radioactive isotope. ^{125}I is the radioactive isotope. The specific activity of the anti-light chain reagent used was in the range 150-200 µC/µg. The cells were spun through 2 FCS gradients and incubated in medium for 14-19 hours at 4°C before transfer.

c) Anti-SRBC PFC were assayed on day 8 after cell transfer following antigenic challenges with 2×10^8 SRBC on days 2 and 5 after cell transfer.

d) Not done.

Table 2.11: The effect of removal of Ig positive rosette forming cells on the ability of thymus cells to collaborate in the anti-hapten response to DNP-flagellin in vitro

Cells cultured [a]	Anti-DNP PFC per culture [b]
Untreated spleen cells	1703 ± 93
Anti-θ ascitic fluid and C' treated spleen	443 ± 44
Anti-θ treated spleen + Thymus cells control rosetted	806 ± 30
Anti-θ treated spleen + Thymus cells depleted of Ig rosette-forming cells [c]	716 ± 77
Thymus, control rosetted alone	<5
Thymus, Ig depleted alone	<5

a) 5×10^6 viable untreated and anti-θ treated spleen cells and 2.5×10^6 Ig depleted and control rosetted thymus cells were cultured per well (final volume, 2.5 ml) as above, with purified, oxidized DNP-MON at a final concentration of 100 ng/ml. CBA/H mice were used in this experiment.

b) Anti-DNP PFC were measured on day 3 after culture. Each value is the arithmetic mean of 4 cultures ± the standard error of the mean.

c) Rosettes were formed with the Ig positive cells and were separated from the non-rosetting lymphocytes by centrifugation on Isopaque/Ficoll. The rosettes sank with the red cells and the non-rosetting cells floated. The procedure was >97% efficient at removing the Ig bearing cells detected by radioautography (Chapter 5).

A separation procedure for depleting lymphoid populations of Ig positive rosette forming cells will be described in Chapter 5 and in Chapters 3 and 4 an *in vitro* anti-hapten response to DNP-flagellin (DNP-MON) is characterized. The response to DNP-MON is T cell dependent, and anti-θ treated cells give a 3-7 fold lower response compared to untreated spleen cells. The response of an anti-θ treated spleen population is partially restored, by addition of thymus cells (3.3.2.3.B). This was confirmed in the experiment reported in Table 2.11 and the effect of removal of the Ig positive rosette forming cells from thymus cells on their ability to restore the anti-DNP PFC response was investigated. No decrease in their ability to help in the anti-DNP response was observed (Table 2.11).

2.4 DISCUSSION

The question posed initially in this chapter was: Do thymus cells contain Ig and if so, how much? The answer was that only a small proportion of thymus cells (<1%) appeared to contain a detectable amount of Ig and within this small proportion there was a hierarchy of anti-light chain-binding cells. Most of the positive cells bound only small amounts of the anti-light chain reagent but a few bound as much as peripheral B cells.

This conclusion was reached using three complementary techniques:-

a) An estimation of the total amount of ^{125}I-anti-light chain Ig, which bound to intact cells by counting in a spectrometer.
b) Radioautography of normal and cortisone resistant thymus cell populations after reaction with the radioactively labelled anti-mouse reagent.
c) An estimation of the total amount of Ig present in cells, using a quantitative microprecipitin inhibition technique in which the inhibitor was total Ig present in cell extracts.

The validity of the conclusion drawn from the first two approaches depends upon the availability of membrane bound Ig to the anti-light chain reagent. For this purpose,

a reagent which had been shown previously to interfere with the specific "suicide" of peripheral T cells by radioactive antigen was used.

The third approach depends on the efficiency of the detergent, nonidet-P-40 to extract and solubilize Ig present in or on the cells. Use of NP-40 by us and by Ey (1973) on preparations of B lymphocytes yielded an estimate of the amount of Ig present on B cells which was comparable to those obtained by other workers using different techniques (Unanue et al., 1971; Eskeland et al., 1971). In addition, Grey et al. (1972b) compared the efficiencies of various procedures for extracting Ig from thymocytes and concluded that solubilization with NP-40 was the most efficient technique of those tried, which included freeze-thawing and urea/acetic acid treatment. However, there are contrary claims (Marchalonis and Cone, 1973).

Attempts to use deoxycholate as an alternative solvent to NP-40 were unsuccessful for technical reasons associated with the microprecipitin inhibition technique used.

The small proportion of Ig positive cells found were looked at in some detail. The first question asked was whether they were intrinsic to the thymus or merely casual contaminants.

Evidence presented indicated that they were present in all strains of mice tested at various ages and were not casual contaminants from blood or from parathymic lymph nodes. The possibility of their selective accumulation in the thymus from the blood was not excluded.

Secondly, what were the nature and properties of these cells? Morphologically, the more heavily labelled cells were small lymphocyte-like cells. The lightly labelled cells were a heterogeneous group of larger cells, some with a high proportion, relative to the nucleus, of basophilic cytoplasm. Some of these may have been plasma cells, but this kind of cell was rare and greatly outnumbered by the other Ig positive cells.

A small proportion (20%) mainly the lightly labelled cells were lysed by anti-θ plus complement. A negligible proportion only was lysed by an anti-B cell, anti-macrophage

antiserum. This antiserum was able to kill 95% of splenic plaque-forming cells and hence it seems likely that few, if any, of the Ig positive cells were plasma cells. It is of interest that Vitetta et al. (1973) have found that synthesis and secretion of Ig by cells in the thymus can be inhibited by an anti-plasma cell serum, suggesting the presence of plasma cells in thymus. In addition Perkins et al. (1972) using electron microscopy radioautography have reported finding small numbers (1/300) of Ig positive cells in thymus which in contrast to splenic B cells had unique morphological characteristics which included fewer ribosomes and a higher cytoplasmic to nuclear ratio (1:3).

Thus the identity of the majority of binding cells is uncertain, although it is known that a proportion of peripheral T cells are rather resistant to anti-θ antibody (Raff and Cantor, 1971).

What is the role of these cells in the thymus? They were considerably enriched in the cortisone resistant fraction which is known to contain most of the immunocompetent cells for activities such as graft vs. host reaction, (Warner, 1964; Blomgren and Andersson, 1969) and helper function (Cohen and Claman, 1971). One possibility is that they are the end product of a differentiation process concerned with the maturation of thymus cells for the peripheral T population. The absence of large amounts of Ig on peripheral T cells argues against this, however, as do the experiments designed to investigate the functional significance of the Ig positive cells. Their removal by an Ig rosette forming cell depletion procedure did not impair the ability of the remaining cells to collaborate in the response to DNP-flagellin in vitro, nor did their removal by radioactive anti-light chain suicide destroy their ability to help in an anti-SRBC response in vivo.

Another possibility is that they are incoming cells from the bone marrow which apparently differentiate in such a way as to lose most of their surface Ig and gain the θ antigen. Hemmingson (1972) has shown that bursal cells can migrate to the thymus. This would explain the lack of susceptibility of the heavy binding cells to anti-θ and the susceptibility of the lightly labelled cells.

A third possibility, that the Ig detected is cytophilic for a minor cell population cannot be discounted (Chapter 5), particularly as a substantial amount appeared to be IgG.

Although it is not possible to quantitate the radioautographic data when such a pronounced hierarchy of binding cells is observed, consideration of the data from the three approaches leads to a consistent picture. This is, that the majority of cells in the thymus contain little Ig, but that there is a minor sub-population which does contain demonstrable Ig. Of this sub-population, a small proportion contain large amounts of Ig, comparable to that present on B cells. It is of interest that of a number of thymomas examined recently (Harris et al., 1973) some were found to contain large amounts of Ig, others little if any. The Ig positive subpopulation present in thymus will be discussed further in Chapter 8.

2.5 SUMMARY

Thymus cells were examined for Ig by exposing them to purified anti-mouse light chain Ig labelled with ^{125}I and examining the amount of reagent bound by bulk counting and radioautography. In addition cell extracts were assayed for total Ig using a quantitative microprecipitin inhibition technique. It was concluded that the majority of cells in thymus contained little Ig but that there was a minor sub-population, of varying morphology, which did contain demonstrable Ig. This small proportion of cells (~1%) appeared to be intrinsic to the thymus and dose response data indicated that there was a hierarchy of Ig positive cells. They were enriched for in the cortisone resistant fraction. The majority were resistant to both anti-θ and an anti-B-anti-macrophage serum, leaving their identity in doubt. Some further properties and attempts to attribute a functional significance to such cells were described.

CHAPTER 3

Characterization of *in vitro* anti-hapten responses

3.1 INTRODUCTION

Following failure to find large amounts of Ig on thymus cells (Chapter 2), systems for measuring functional activity of peripheral T cells were investigated. It was planned to use such systems for looking further at properties of T cells, the nature of the T cell receptor and the significance of Ig on the T cell surface. *In vitro* assay systems were preferred as they had a number of advantages over *in vivo* systems.

For example: a) fewer animals were required for each experiment; b) results were obtained more quickly (3-4 days compared to weeks); c) systems were more open to analysis *in vitro*; and d) variables in the experiments were more easily controlled.

Several major biological activities have been attributed to T cells:-

a) the rejection of "foreign" cells, which may be grafted cells, tumours or altered cells (e.g. virus infected cells).
b) the elaboration of a variety of non-antigen specific factors (lymphokines) on activation by antigen; e.g. migration inhibition factors (MIF), chemotactic factors, cytotoxic factors and mitogenic factors. Many of these factors affect the behaviour of macrophages or their precursors.
c) collaboration with B cells either to enhance or suppress antibody formation.

These functions of T cells were considered for *in vitro* assay systems.

In vitro cytotoxic assays provide the only direct measure of T cell function. Assays for measuring the activity of cytotoxic T cells for viral infected target cells were developed and characterized by workers in this school (Gardner et al., 1974 a, b; Doherty et al., 1974a, b). T cells activated to ectromelia virus or lymphocytic choriomeningitis virus *in vivo* were able to release ^{51}Cr from viral infected target cells. The release appeared to be viral specific, as T cells activated to one virus would only release ^{51}Cr from

targets infected with the same virus. Attempts were made (in collaboration with A. Hapel) to set up a cytotoxicity assay for a simpler virus, the Group A arbovirus, Semliki Forest virus. This virus consists of 3 proteins, all of which can be readily purified. However, no reproducible, specific lysis above levels obtained by immune spleen cells primed to a heterologous virus could be obtained on a number of target systems tested. Also, the nature of the specificity of the killing systems (i.e. whether the T cell recognises the virus or a virus-induced altered H-2 region or both, Zinkernagel and Doherty, 1974) and the relevance of these systems to the in vivo activity of the T cell is not yet clear.

T-B collaboration in antibody formation has, however, been clearly demonstrated both in vivo and in vitro (see 1.2) and is a well recognized and important T cell function even though measurement of T activity is indirect, as it is based on the final activity of other cells. In addition, one of the strongest pieces of evidence that Ig is the functional T cell receptor has come from the work of Feldmann and colleagues (1972c, 1973) who claimed that a soluble Ig-antigen complex released from T cells could substitute for the helper function of T cells in the response to hapten-carrier antigens in vitro. In this chapter, therefore, in vitro anti-hapten responses to DNP-HCY, DNP-HGG and DNP-MON are described and two of these, the response to DNP-HCY and DNP-MON are characterized in some detail. Future chapters report experiments using both these culture systems to characterize a procedure for separating mouse T and B lymphocytes (Chapter 5) and to investigate mechanisms of T-B collaboration (Chapters 6 and 7).

3.2 MATERIALS AND METHODS

3.2.1 Animals

CBA/J mice were purchased from Jackson Laboratories, Bar Harbour, Maine, U.S.A. Other mice used were obtained from breeding colonies in this School. Nude mice were from both heterozygous (nu+ x nu+) and homozygous (nu+ x nu/nu) matings of mice obtained originally from Dr. M. Holmes of the Walter and Eliza Hall Institute, Melbourne. The heterozygous nudes,

originally outbreds, had been backcrossed in CBA/H and Balb c mice. Mice were used at 7-9 weeks of age. ATxBM (adult thymectomized, irradiated, bone marrow reconstituted) mice were thymectomized at 7 weeks of age, lethally irradiated (850 rads) 2 weeks later and reconstituted by i.v. injection of 2 x 10^7 bone marrow cells. Unless otherwise stated CBA/H mice were used throughout. Outbred New Zealand rabbits 6-12 months of age were used for raising antisera.

3.2.2 Irradiation of mice

Groups of 5 mice were placed in cylindrical plastic containers (diameter 9.5 cm , height 4.5 cm) on rotating stages 15 cm from a ^{60}Co rod source and given 850 rads whole body gamma irradiation, over a period of 20-25 min.

3.2.3 Antigens

Monomeric flagellin (MON), molecular weight 40000, was prepared from the flagella of Salmonella typhimurium SL 870 (Ada et al., 1964) and oxidized to prevent repolymerization (Parish and Stanley, 1972).

Haemocyanin (HCY), a pentamer of molecular weight 450000, was crystallized from the haemolymph of the South Australian crayfish, Jasus lalandii. (Moore et al., 1968). Human gamma globulin (HGG), Fraction II, was purchased from CSL, Melbourne and bovine serum albumin (BSA), Fraction V from Armour Pharmaceutical Co., England. These proteins were dinitrophenylated, according to the method of Eisen, (1964). Conjugation ratios used were $DNP_{0.25}$ MON to $DNP_{2.5}$ MON, DNP_{48} HCY, DNP_{20} HGG and DNP_{20} BSA. SRBC were obtained from a single sheep in this school, and were stored in Alsever's solution and washed 4x in saline prior to use.

Mice were primed with 10 µg MON in saline i.v., 1 week to 1 month before use, 1 mg HCY or 500 µg alum precipitated HGG, with 10^9 B. pertussis organisms (Commonwealth Serum Laboratories (CSL), Melbourne) in 0.2 ml saline, i.p., 1 week before use; or 500 µg alum precipitated DNP-BSA and pertussis i.p., as above, 2 to 4 months before use.

3.2.4 Preparation of cell suspensions

Spleen and thymus cell suspensions were prepared

in tissue culture medium, under sterile conditions as described earlier (2.2.2). The viability of such cell suspensions was generally >90%. Cortisone resistant thymocytes were prepared from the thymuses of mice which had received cortisyl acetate (5mg/0.2ml) s.c. 24 hours previously (2.3.9).

3.2.5 Tissue culture

Eagle's minimal essential medium (F 15) (Grand Island Biological Co., Grand Island, New York) buffered with sodium bicarbonate and supplemented with 10% foetal calf serum (CSL, Melbourne), 100 µg/ml streptomycin, 100 units/ml penicillin G, and 10^{-4} M mercaptoethanol was used for cell culture. Cells were cultured with antigen in this medium (5-6 x 10^6 cells/well in a final volume of 2.5 ml) in 16 mm Linbro trays (Linbro Chemical Co., New Haven, Connecticut, U.S.A.).

Cultures were placed in a humidified incubator at 37°C in an atmosphere of 10% CO_2, 7% O_2 in N_2, for three days, and then harvested and assayed for plaque-forming cells.

3.2.6 ^{3}HTdr uptake in vitro

Cells (5 x 10^6/2.5ml) were incubated in culture medium, as above, for 3-5 days with antigen ^{3}HTdr (Radiochemical Centre, Amersham, U.K.) was then added for 5 h and the uptake into DNA measured both by bulk counting and by radioautography.

3.2.7 Radioautographic procedure

See 2.2.6

3.2.8 Preparation of hyperimmune anti-SRBC antiserum

Rabbits were injected i.v. (ear) twice a week with 1ml of a suspension of washed SRBC with a concentration gradually increasing from 10 up to 50 per cent SRBC in saline. Seven days after the sixth injection, the animals were bled and serum collected. Anti-SRBC antibody titres were assayed by a haemagglutination procedure (Langman, 1972) and were generally greater than 1:1500.

3.2.9 Precipitation of anti-SRBC globulin

Globulins were precipitated from the immune serum by ammonium sulphate precipitation (35% saturation). After washing and reprecipitation the globulins were dissolved in 0.1M phosphate, 0.002M EDTA buffer and dialyzed against the same overnight.

3.2.10 Preparation of anti-SRBC Fab

Anti-SRBC globulins prepared as above, were digested with papain (2x crystallized Cat. No. P3125 Sigma Chemical Co.) for 2-4 h at 37°C according to Porter (1959). The reaction was stopped by addition of solid iodoacetamide and the mixture cooled in ice and then dialysed (overnight at 4°C) against Dulbecco's balanced salt solution (DBSS) (Dulbecco and Vogt, 1954). This procedure allowed most of the Fc piece to precipitate.

3.2.11 Removal of undigested IgG from Fab preparation

As the reaction with papain was not 100% efficient, there was still some undigested IgG present in the Fab reagent. It was necessary to remove this as the Fc portion of the IgG was able to fix guinea pig complement and cause lysis of the coated SRBC in the plaque assay. The Fab reagent was purified in two ways - either by centrifugation on preparative (36 ml) sucrose gradients (10-30% sucrose in DBSS + 15mM NaN_3, 55K, 24-30h), or by passage through a G-200 column (approx. 90 cm long with an internal diameter of 2.5 cm). Both procedures achieved good separations and enabled the Fab reagent to be obtained in pure form.

3.2.12 Dinitrophenylation of the anti-SRBC Fab

The Fab reagent was dinitrophenylated, as for the protein antigens, using 2, 4-dinitrobenzene sulphonate (Eastman Organic Chemicals, N.Y.) as described by Strausbauch et al., (1970). An average number of 3 DNP groups per Fab molecule was determined spectrophotometrically (Eisen, 1964).

3.2.13 Reaction of SRBC with anti-SRBC Fab-DNP

The anti-SRBC Fab-DNP was reacted at various concentrations with 10% SRBC in DBSS (1h, 37°C), followed by

2 washes in DBSS. The optimum dilution of reagent was the one which detected the greatest number of clear, regular plaques.

3.2.14 Enumeration of plaque-forming cells

Plaque forming cells (PFC) were detected using the technique of Cunningham and Szenberg, (1968). To detect anti-DNP PFC, SRBC were coated with the dinitrophenylated rabbit anti-SRBC Fab as described above. Indirect plaques were developed using a rabbit anti-mouse Ig serum. This was prepared by injecting rabbits with 1 mg of mouse Ig (40% $(NH_4)_2SO_4$ precipitate of normal mouse serum) in FCA into the flanks at 4 sites. Four weeks later the rabbits received a second challenge in FIA (1 mg, 4 sites in flanks) and one week later serum was obtained and tested. The optimum dilution of developing antiserum (i.e. the one which detected most indirect plaques) was obtained by titrating it in a plaque assay.

The tissue culture response was largely IgM in nature, so only direct plaques were measured in the *in vitro* experiments.

3.2.15 Preparation of anti-θ reagent

Anti-θ ascitic fluid was prepared in AKR/J mice which were given up to 10 intraperitoneal injections of 5-10 x 10^7 CBA thymocytes at weekly intervals. The first injection was given with 10^9 *B. pertussis* organisms. Three days before the final dose of thymocytes, the mice were injected intraperitoneally with 0.2 ml of a 10% suspension of sarcoma 180 cells in saline (Tikasingh et al., 1966). The ascitic fluid was harvested 7 days after the final thymocyte injection. Unimmunized AKR/J mice were used as donors of control ascitic fluid. The anti-θ ascitic fluid was cytotoxic for >98% of thymus cells and for 20 to 30% of spleen cells.

3.2.16 Treatment of cells with anti-θ and complement

Cells were incubated (37°C, 30 min) at a concentration of 5 x 10^7 - 1 x 10^8 cells/ml with a 1:2 dilution of anti-θ ascitic fluid, washed and resuspended in a 1:2 dilution of guinea pig serum, adsorbed previously on agarose

(80 mg/ml, 0°C for 1 hour) and checked for anti-mouse activity, as even after this adsorption some batches of complement were found to have considerable toxicity for mouse thymus cells. Cells were then incubated for a further 20 min at 37°C, washed and recounted. Viability was judged by trypan blue (0.05%) dye exclusion.

3.2.17 Treatment of cells with anti-Ig serum and complement

Anti-IgM serum was kindly donated by P.L. Ey (2.3.2.5). After adsorption with thymocytes (5 x 10^8 cells/ml) this antiserum was cytotoxic for 40-50% of spleen cells and less than 10% thymus cells were affected. Cells were treated with this antiserum as per 3.2.16. After the reaction dead cells were removed by mixing cells with 20 times their volume of isotonic glucose and passing them through cotton wool. Cells recovered were >95% viable.

3.2.18 Fractionation of spleen cells on Isopaque/Ficoll

Spleen cells were depleted of immunoglobulin bearing cells by the one-step fractionation method developed by Parish and Hayward (1974) for the rat. The procedure for the mouse is described in detail in a later chapter (Chapter 5).

3.2.19 Footpad assay of delayed type hypersensitivity (DTH)

Mice were injected subcutaneously behind the left and right shoulders with antigen in a total of 0.2 ml saline or 0.2 ml complete Freund's adjuvant (Difco, Detroit, Michigan, U.S.A.) and their hind footpads were assayed for DTH reactivity 6 days after this injection. The dorso-ventral thickness of each hind footpad was measured in tenths of a millimetre (±0.05 mm) with a dial gauge caliper ("Schnelltaster", H.C. Kröplin, Hessen, West Germany) before and after the subcutaneous injection of antigen and saline into the right and left footpads respectively, using a micrometer syringe and the mean percentage increase in footpad thickness calculated (Cooper, 1972a).

3.3 RESULTS

3.3.1 Measurement of anti-DNP antibody

3.3.1.1 Preliminary remarks

Before in vivo or in vitro systems for measuring T cell helper function in an anti-hapten response could be set up, it was necessary to have a reproducible, reliable and quick method for assaying anti-hapten antibody. The most common method for measuring serum anti-hapten antibody involved modifications of the Farr assay (Brownstone et al., 1966; Mitchison, 1971; Roelants and Askonas, 1971; Hamaoka et al., 1973). This method relied on reaction of the serum under test with ^{125}I-labelled DNP peptides, followed by precipitation of the antigen-antibody complexes with ammonium sulphate and subsequent counting of the supernatants. For measuring anti-DNP PFC, DNP had to be coupled to SRBC. This could be done either directly by chemical means (Layson and Sehon, 1967; Yamada and Yamada, 1969; Havas and Hraba, 1969; Trump, 1972), or indirectly using a dinitrophenylated rabbit anti-sheep reagent (e.g. Strausbauch et al., 1970; Miller and Segre, 1972) or a chicken anti-sheep reagent (Silver, et al., 1971). The chicken anti-sheep reagent had the advantage that chicken IgG did not fix guinea pig complement so that the antiserum could be coupled directly with the hapten and used at a sub-agglutinating dose for coating SRBC. With the rabbit reagent, however, unless very low doses of reagent could be used (Miller and Segre, 1972), the Fab of the Ig had to be made to prevent lysis of the SRBC by guinea pig complement during the plaque assay.

All the above methods yielded similar results as regards the kinetics of the anti-DNP response in vivo, but the overall number of plaques detected varied. The indirect coating methods seemed more efficient at detecting anti-DNP PFC. In addition, indirectly coated cells had the advantage of being stable for longer periods after coating.

A direct method for coating SRBC, described by Trump, (1972) in which SRBC were reacted with FDNB was tried with success. The method had the advantage that large numbers of coated SRBC could be prepared at one time. However, the method had a number of disadvantages:

a) the coating procedure was not reliably reproducible and seemed to depend quite critically on parameters such as

the temperature of the reaction (room temperature variations) and the state, e.g. age, of the SRBC being coated;

b) the coated cells were not reproducibly stable. Some batches would last no more than 1-2 days while others could be stable for a week or more;

c) no indirect plaques were ever detected using these directly coated cells under conditions known to detect indirect plaques for the anti-SRBC response.

An indirect method of coating SRBC with DNP using anti-SRBC-Fab-DNP was much more successful and satisfied criteria of being quick, reproducible and reliable. Details of the method are described by Strausbauch et al., (1970) and in the Materials and Methods section of this chapter. The procedure had the advantages that:-

a) the numbers of plaques detected were consistent with those described by others using indirect procedures (i.e. of the order of 10^5 PFC per spleen at the peak of response);

b) plaques were clearly defined, regular and easy to count;

c) indirect plaques were readily detected with developing antiserum;

d) coated SRBC were reliably stable for 1-2 weeks.

This method was thus adopted and used in all future experiments where anti-DNP PFC were measured.

3.3.1.2 Properties of the anti-DNP PFC detected using anti-SRBC Fab-DNP coated SRBC

A. Appearance of anti-DNP PFC

Plaques obtained with the optimal dilution of anti-SRBC Fab-DNP were clear, circular (1-3 mm diameter) and easily counted. Under high magnification a central lymphoid cell was clearly visible and RBC ghosts could be seen in the plaque.

B. Stability of the coated SRBC

The same number of background anti-SRBC PFC (around 100 per spleen) were obtained on both DNP coated and normal SRBC.

C. Specificity of the anti-DNP PFC

When dilutions of DNP-BSA were incorporated into the plaque assay an inhibition of the anti-DNP PFC response was observed (Table 3.1). This inhibition was not due to any toxic properties of the DNP-BSA for PFC as at the same dilutions at which it was inhibitory for the anti-DNP PFC response it had no effect on the ability of an immune anti-SRBC spleen cell suspension to form plaques on SRBC.

Dilution of the anti-DNP immune spleen cell suspension caused a corresponding decrease in the number of plaques observed.

D. Kinetics of the response and detection of indirect PFC

Unimmunized mice gave only background numbers of plaques (100-200 per spleen). Following immunization with 500 µg of alum precipitated DNP-BSA and 10^9 B. pertussis organisms i.p., anti-DNP PFC increased in number through to the 6th day and were found in decreasing numbers in the spleen after the 7th day. Indirect plaques were found in low numbers (10-60% of the direct response) from day 4 onwards. The secondary response was predominantly indirect and tended to peak at day 4 after challenge (Table 3.2).

3.3.2 Characterization of in vitro anti-hapten responses

Systems for obtaining in vitro anti-DNP responses to hapten-carrier antigens, DNP-HCY, DNP-HGG and DNP-MON were set up and two of these, the responses to DNP-HCY and DNP-MON, were characterized in some detail.

3.3.2.1 Characterization of the anti-hapten response to DNP-HCY

A. Kinetics of the response

When spleen cells from normal CBA/H mice were cultured with DNP-HCY only a minimal response was observed unless cells primed to both the hapten and carrier were used. Initially, hapten primed cells (2×10^6 per well) were cultured with increasing numbers of carrier primed cells (1×10^6 - 4×10^6 per well) and the response compared with hapten primed cells cultured with 4×10^6 normal (i.e. umprimed) spleen cells. Within each group there were four

Table 3.1: Specificity of the anti-DNP PFC

Inhibitor (DNP-BSA) present in plaque assay a)	Anti DNP PFC per spleen b)	Anti SRBC PFC per spleen c)
No inhibitor	2440	2620
Inhibitor undiluted	180	1880
Inhibitor 1/2 dilution	200	2160
Inhibitor 1/5 dilution	760	2280
Inhibitor 1/10 dilution	720	2680

a) 1 drop of inhibitor or the appropriate dilution was added to the plaque mixture (0.1 ml spleen cells, 2 drops medium, 2 drops SRBC, 1 drop C' where 1 drop was equivalent to 0.01 ml).

b) Spleens were taken on day 3 after 1^{o} challenge (500 µg DNP-BSA alum precipitated + 10^{9} B. pertussis organisms i.p.).

c) Spleens were taken on day 3 after primary challenge with 2×10^{8} SRBC i.v.

Only direct plaques were measured.

Table 3.2: Development of indirect anti-DNP PFC

Developing antiserum present in plaque assay a)	Anti-DNP PFC per spleen Primary response b)	Secondary response c)
No developing serum	840	2160
Serum 1/20	360	12080
Serum 1/40	1260	13440
Serum 1/80	1240	11520
Serum 1/160	1120	6160
Serum 1/320	860	3960
Serum 1/640	790	2360
Serum 1/1280	820	2280

a) 1 drop of developing serum or appropriate dilution per plaque assay.

b) Spleens were taken on day 5 after primary challenge (500 µg alum precipitated DNP-BSA + 10^9 *B. pertussis* organisms i.p.).

c) Spleens were taken on day 5 after 2^o challenge (as for 1^o).

Dilution 1/40 was optimal for developing indirect plaques.

replicate wells. Cultures were set up in triplicate so that the response could be followed on three successive days, days 3, 4 and 5 after culture. DNP-HCY was at a final concentration of 2 µg/ml, a fortuitous choice as the optimum concentration of antigen was later found to be between 2-3 µg/ml (Table 3.3). The anti-DNP response of such cultures is summarized in Figure 3.1.

The wells which received carrier primed cells showed significantly higher anti-DNP responses compared to those which received unprimed cells, on all 3 days of culture. The optimal PFC response and difference occurred on day 4, (open circles and triangles), the background plaques (i.e. where no carrier primed cells were added) remaining at the same level on days 3 and 4, whereas cultures which received HCY primed cells continued to show an increase in the anti-DNP PFC. By day 5 the PFC response had severely declined in all groups.

B. Optimum culture conditions

The difference in anti-DNP PFC response between cultures receiving carrier primed cells rather than normal spleen cells was only 2½ fold, and it was hoped that by investigating parameters of the tissue culture system larger differences might be obtained. Three such parameters were antigen dose, replenishment of culture medium and the time between carrier priming and cell culture.

Antigen dose

Hapten primed cells (2×10^6 per well) were cultured with carrier primed (4×10^6 per well) or unprimed spleen cells (4×10^6 per well) with varying concentrations of DNP-HCY and assayed for anti-DNP PFC on day 4. The results are shown in Table 3.3. The optimal antigen dose was clearly 2.5 µg/ml where again a 2½ fold increase in the anti-DNP response was obtained in the presence of carrier primed cells.

Effect of change of medium during culture

The effect of replenishing the culture medium on the anti-DNP PFC response was investigated. Cells were cultured as usual and either left untouched during culture or were

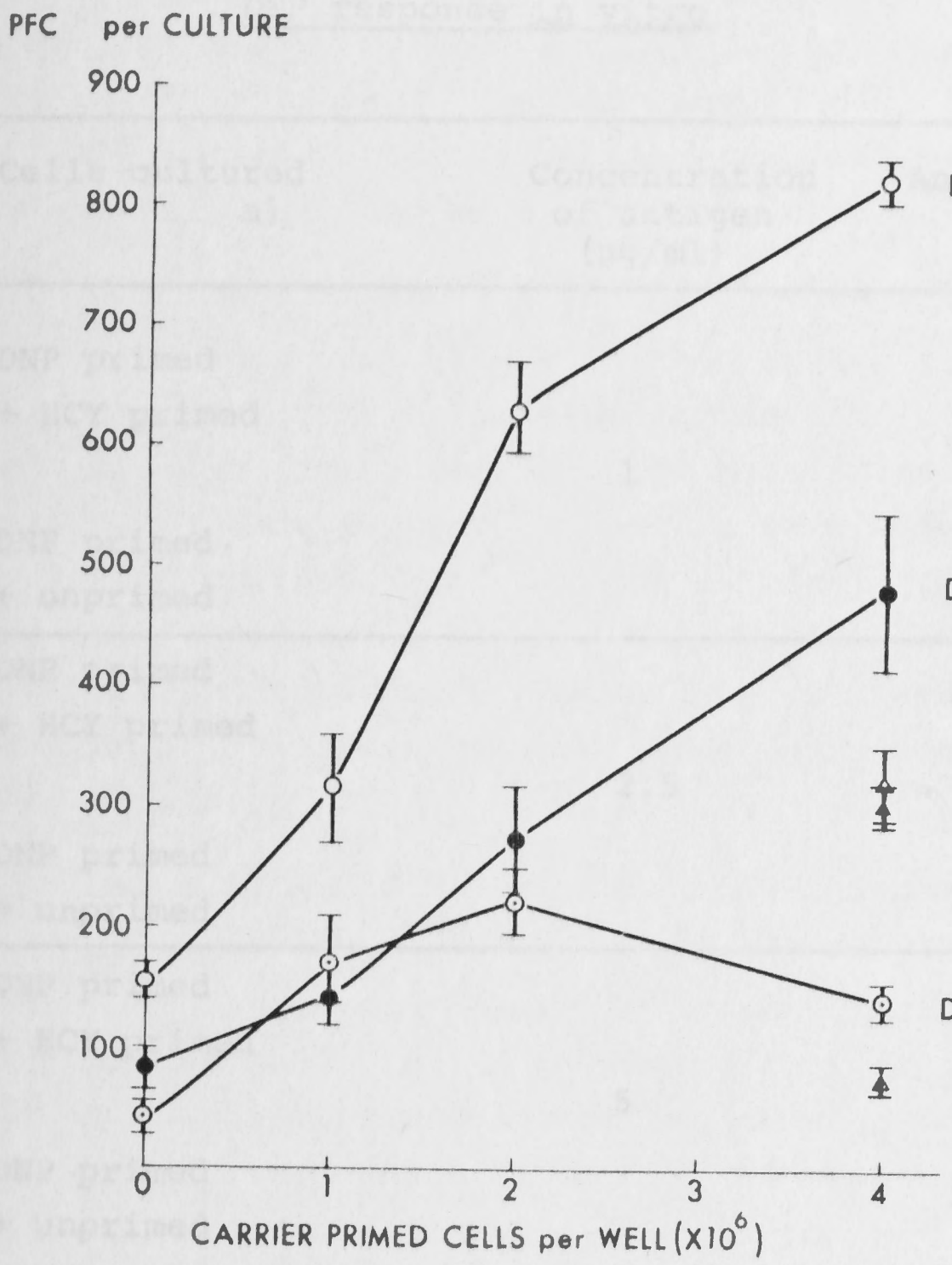


Figure 3.1 The in vitro anti-DNP response to DNP-HCY. Hapten primed cells ($2x10^6$ per well) were cultured with increasing numbers of carrier primed cells (6 days after priming - $1x10^6$ - $4x10^6$ per well) (Circles), or with normal, unprimed spleen cells ($4x10^6$ per well) (triangles). The response to DNP was measured on days 3, 4 and 5 after culture. DNP-HCY was at a final concentration of 2µg/ml.
Medium and antigen were replaced on days 2 and 4 of culture. Each point represents the arithmetic mean of four wells ± the standard error of the mean.

Table 3.3: Effect of concentration of DNP-HCY on the anti-DNP response in vitro

Cells cultured a)	Concentration of antigen (µg/ml)	Anti-DNP PFC per culture b)
DNP primed + HCY primed	1	522 ± 51
DNP primed + unprimed	1	300 ± 30
DNP primed + HCY primed	2.5	681 ± 39
DNP primed + unprimed	2.5	258 ± 39
DNP primed + HCY primed	5	228 ± 24
DNP primed + unprimed	5	186 ± 3

a) 2×10^6 DNP primed cells were cultured with 4×10^6 carrier primed (6 days previously) or unprimed cells. Medium and antigen were replaced on day 2.

b) Cultures were assayed on day 4 of culture. Each value represents the arithmetic mean of 4 wells ± the standard error of the mean.

given a change of medium with fresh antigen on day 2 or a change of medium without antigen on day 2 or day 3. Results are given in Table 3.4. There was little difference in the overall anti-DNP response or the response over background levels following any of these procedures.

Effect of time of carrier priming on the anti-DNP response

To investigate when the carrier primed population was most efficient at helping in the anti-DNP response, spleen cells from mice which had been primed at intervals from 3 to 36 days prior to culture were added to DNP primed cells and cultured with DNP-HCY as usual. The responses of two such experiments are shown in Table 3.5. There was some slight trend towards increased help at longer periods after priming but differences above background were still no more than 3 fold.

C. Dose response of carrier primed cells and specificity of the help effect

The optimum number of carrier primed cells in the culture was investigated by adding increasing numbers (1×10^6 to 8×10^6) of carrier primed or unprimed spleen cells to hapten primed cells (2×10^6) and culturing them under optimal conditions (3.3.2.1 B). At the ratio of 2×10^6 hapten primed cells to 5×10^6 carrier primed cells the best response was observed - a 3 fold difference above the background level (Figure 3.2). It is of interest that hapten primed cells and carrier primed cells, in the absence of antigen (open triangles), gave a background anti-DNP response of the same order as that of the unprimed cells. It was thus likely that all the response above background was a specific help effect.

To show that it was a specific effect and not just a consequence of recent priming, allowing, for example, more efficient handling of antigen, the experiment shown in Figure 3.2 was repeated. This time, however, heterologously primed spleen cells were included as well as normal cells as a specificity control for the help effect. Results are given in Table 3.6. Cultures which received HGG primed spleen cells gave slightly higher background responses than cultures with unprimed cells, but responses obtained when HCY primed cells

Table 3.4: Effect of change of medium on the anti-DNP response to DNP-HCY

Cells cultured a)	Medium change	Anti-DNP PFC per culture b)
DNP primed	None	319 ± 72
DNP primed + HCY primed	None	575 ± 57
DNP primed + unprimed	None	317 ± 29
DNP primed	Medium change + antigen day 2	111 ± 20
DNP primed + HCY primed	Medium change + antigen day 2	535 ± 27
DNP primed + unprimed	Medium change + antigen day 2	237 ± 30
DNP primed	Medium change - antigen day 2	123 ± 42
DNP primed + HCY primed	Medium change - antigen day 2	490 ± 61
DNP primed + unprimed	Medium change - antigen day 2	209 ± 15
DNP primed	Medium change - antigen day 3	162 ± 25
DNP primed + HCY primed	Medium change - antigen day 3	574 ± 25
DNP primed + unprimed	Medium change - antigen day 3	297 ± 52

a) 2×10^6 DNP primed cells were cultured with 4×10^6 carrier primed (6 days previously) or unprimed cells. DNP-HCY was at 2.5 µg/ml.

b) Cultures were assayed on day 4 of culture. Each value represents the arithmetic mean of 4 wells ± the standard error of the mean.

Table 3.5: The effect of time after carrier priming on the ability of spleen cells to help in an anti-DNP response

Days after priming	Anti-DNP PFC per culture [b]			
	Expt. 1		Expt. 2	
	DNP primed + HCY primed	DNP primed [a] + HGG primed	DNP primed + HCY primed	DNP primed +HGG primed
3	n.d. [c]	n.d.	201 ± 11	115 ± 11
5	371 ± 57	199 ± 44	n.d.	n.d.
7	n.d.	n.d.	410 ± 28	143 ± 31
17	558 ± 67	n.d.	524 ± 31	299 ± 67
22	n.d.	n.d.	559 ± 22	374 ± 34
28	704 ± 30	n.d.	840 ± 70	393 ± 34
36	734 ± 28	278 ± 54	n.d.	n.d.

a) 2×10^6 hapten primed cells and 5×10^6 carrier primed cells were cultured per well. DNP-HCY was at a final concentration of 2.5 µg/ml. Cultures were assayed on day 4.

b) Each value is the arithmetic mean of 4 wells ± the standard error of the mean.

c) Not done.

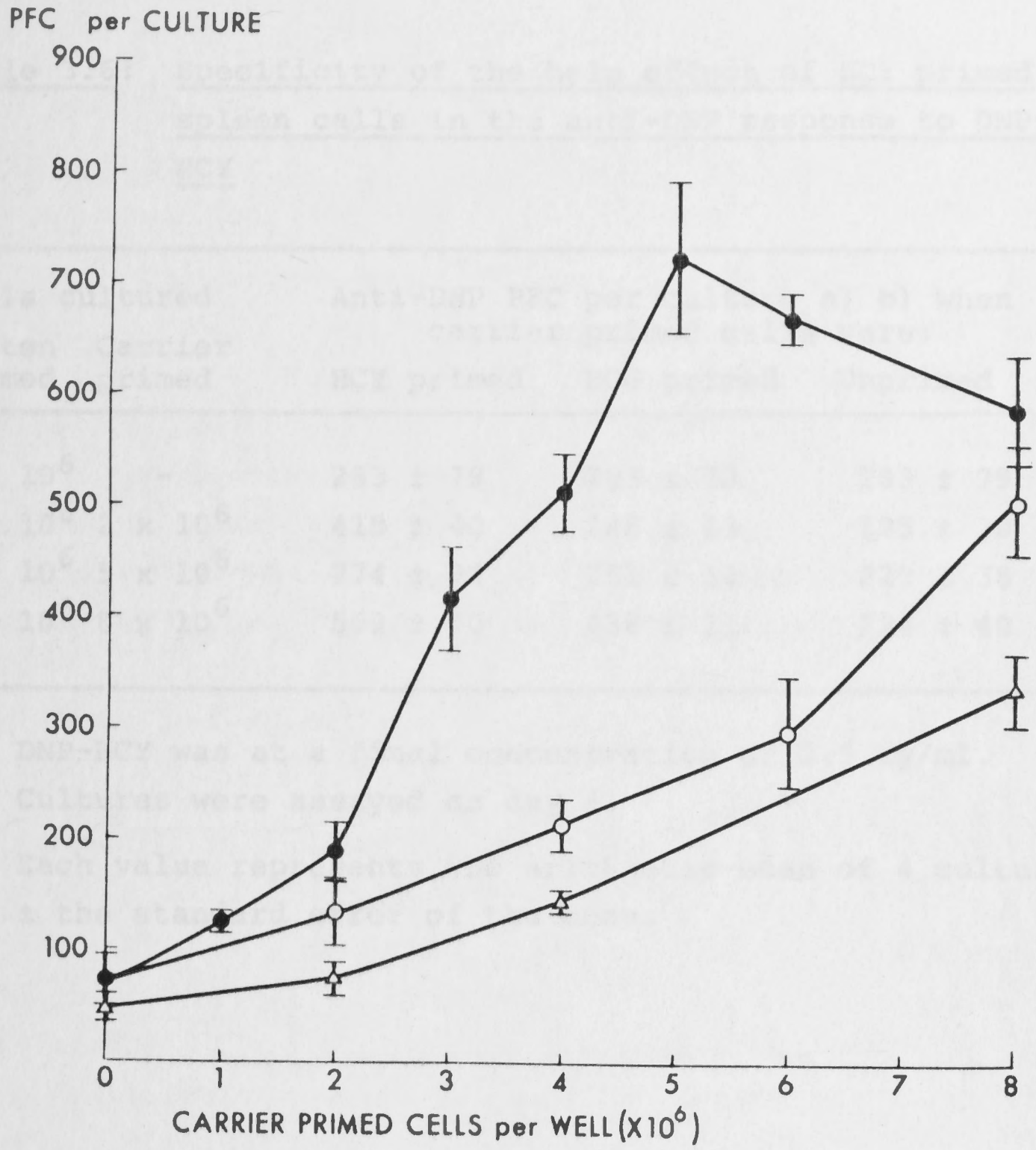


Figure 3.2 The anti-DNP PFC response obtained when increasing numbers of carrier (i.e. HCY primed 6 days previously) or unprimed spleen cells were cultured with DNP primed spleen cells (2×10^6 per well)

●-● DNP primed + HCY primed cells
DNP-HCY at 2 µg/ml.

○-○ DNP primed + unprimed spleen cells
DNP-HCY at 2 µg/ml.

△-△ DNP primed cells + HCY primed cells
No DNP-HCY in cultures.

Cultures were assayed on day 4. Each point represents the arithmetic mean of 4 wells ± the standard error of the mean.

Table 3.6: Specificity of the help effect of HCY primed spleen cells in the anti-DNP response to DNP-HCY

Cells cultured		Anti-DNP PFC per culture a) b) when carrier primed cells were:		
Hapten primed	Carrier primed	HCY primed	HGG primed	Unprimed
2×10^6	-	283 ± 79	283 ± 79	283 ± 79
2×10^6	2×10^6	418 ± 40	286 ± 13	175 ± 20
2×10^6	5×10^6	774 ± 24	351 ± 54	223 ± 38
2×10^6	8×10^6	569 ± 40	438 ± 21	224 ± 40

a) DNP-HCY was at a final concentration of 2.5 µg/ml. Cultures were assayed on day 4.

b) Each value represents the arithmetic mean of 4 cultures ± the standard error of the mean.

were present were clearly better.

D. Collaboration between DNP primed and HCY primed cell populations

The increase in PFC response when HCY primed cells, rather than HGG primed cells, were cultured with the hapten primed cells was due to a true collaborative response between the hapten and the carrier primed spleen populations as shown by some representative experiments in Table 3.7. The additive response obtained by either hapten or carrier primed cells cultured alone was always at least two fold less than the response obtained when the two populations were cultured together, and this was 2-3 fold higher than the response of DNP primed cells cultured with heterologously primed carrier cells.

E. Effect of treatment of the carrier primed cells with anti-θ and anti-Ig and complement

To show that it was primed T cells in the carrier population that were responsible for the collaborative effect, the carrier primed population was treated with anti-θ and complement prior to culture with the hapten primed cells. However, this treatment had variable effects on the anti-DNP PFC response, from complete abrogation of the collaborative effect, to partial abrogation, to no effect (Table 3.8). There was an indication that the anti-θ treatment was more effective on spleen cells primed for periods greater than 6 days. Treatment of the carrier primed population with anti-immunoglobulin serum prior to culture decreased the collaborative effect (Table 3.8). Thus it appeared that both B and T cells in this population were important in the in vitro anti-DNP response to DNP-HCY.

F. Fractionation of carrier primed cells on Isopaque/Ficoll

A role for T cells in the collaborative response was further demonstrated following fractionation of the carrier primed cells on Isopaque/Ficoll (Chapter 5). These cells were depleted of Ig bearing cells and the ability of the upper layer (i.e. T cells) to collaborate with the hapten primed

Table 3.7: Collaboration between DNP primed and HCY primed cell populations

Cells cultured [a]	Anti-DNP PFC per culture [b]			
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
DNP primed	234 ± 14	133 ± 19	133 ± 21	120 ± 6
HCY primed	94 ± 12	141 ± 15	134 ± 38	31 ± 5
DNP primed + HCY primed	604 ± 67	876 ± 57	623 ± 99	593 ± 14
DNP primed + HGG primed	232 ± 4	127 ± 2	264 ± 30	199 ± 8

a) 2×10^6 DNP primed cells were cultured with 5×10^6 HCY or HGG primed spleen cells. DNP-HCY at 2.5 µg/ml.

b) Cultures were assayed on day 4. Each value is the arithmetic mean of 4 wells ± standard error of the mean.

Table 3.8: Effect of treatment of the carrier primed cells with anti-θ or anti-Ig and complement on the anti-DNP response

Cells cultured [a]	Treatment of carrier primed cells	Anti-DNP PFC per culture [e]				
		Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5
DNP primed alone	-	310 ± 45	205 ± 37	133 ± 19	40 ± 16	141 ± 15
DNP primed + HCY primed	Untreated	643 ± 34	578 ± 111	n.d	n.d.	n.d.
DNP primed + HCY primed	Normal ascitic fluid and C'	590 ± 108	590 ± 127	976 ± 57	411 ± 47	715 ± 62
DNP primed + HCY primed	Anti-θ ascitic fluid and C'	528 ± 39	566 ± 69	167 ± 34	238 ± 23	303 ± 45
DNP primed + HCY primed	Anti-Ig and C'	312 ± 51 [b] 195 ± 15 [c] 250 ± 31 [d]	n.d. [f]	n.d.	196 ± 43	n.d.
DNP primed + HGG primed	Untreated	356 ± 47	272 ± 26	127 ± 2	225 ± 17	148 ± 36

a) 2×10^6 DNP primed cells were cultured with 5×10^6 viable carrier primed cells. In experiments 1, 2 and 3 carrier primed cells were taken 6-8 days post priming and in experiments 4 and 5, more than 30 days after priming.

b) 1×10^6, c) 3×10^6 and d) 5×10^6 viable anti-Ig treated carrier primed cells per culture.

e) Cultures were assayed on day 4. DNP-HCY at 2.5 µg/ml. Each value represents the arithmetic mean of four cultures ± the standard error of the mean.

f) Not done.

B cells examined. It was shown (Table 3.9) that the T cells could collaborate to give good anti-hapten responses to DNP-HCY. This collaboration was removed by treating the upper layer cells with anti-θ and complement.

T cells, therefore had a role in the collaborative anti-hapten response to DNP-HCY, but the role of the B cell remained to be investigated. The small differences with the DNP-HCY response hampered further investigations along these lines with this system, but using the *in vitro* response to DNP-MON (3.3.2.3) further light on the role of the carrier specific B cell in collaboration was obtained (Chapters 6 and 7).

G. *Collaboration between DNP primed and HCY primed cells in vivo*

Collaboration between DNP primed and HCY primed cells could also be demonstrated *in vivo* (Table 3.10), when these cells were injected into irradiated recipients (850 rads, 24 h previously) with 100 μg DNP-HCY. Spleens were harvested 7 days after the cell transfer and indirect anti-DNP PFC measured. There was clear collaboration between the two cell populations, as observed *in vitro*.

3.3.2.2 *Response to DNP-HGG in vitro*

Collaboration between DNP primed and HGG primed cells was demonstrated *in vitro* in the anti-DNP response to DNP-HGG. Responses were lower, however, than the anti-DNP-HCY responses as shown in Table 3.11, where optimal conditions for the response were used.

3.3.2.3 *Response to DNP-MON in vitro*

When spleen cells from CBA/H mice were cultured with DNP-MON as in 3.3.2.1A, in contrast to the previous anti-hapten responses (3.3.2.1 and 3.3.2.2), a large

Table 3.9: Fractionation of carrier primed cells on Isopaque/Ficoll - ability of pure T cells to collaborate with hapten primed B cells in the anti-DNP PFC response

Cells cultured [a]	Anti-DNP PFC per culture [b]		
	Expt. 1	Expt. 2	Expt. 3
DNP primed	120 ± 6	234 ± 14	108 ± 3
DNP primed + HCY primed	593 ± 14	800 ± 77	583 ± 31
DNP primed + HGG primed	199 ± 8	232 ± 4	199 ± 8
DNP primed + HCY primed T cells untreated	738 ± 26	528 ± 7	454 ± 25
DNP primed + HCY primed T cells treated with normal ascitic fluid and C'	n.d. [c]	n.d.	378 ± 16
DNP primed + HCY primed T cells treated with anti-θ and C'.	n.d.	n.d.	74 ± 11
HCY primed T cells alone	<5	<5	0

a) 2×10^6 DNP primed cells and 5×10^6 HCY primed spleen cells were cultured per well. 5×10^6 HCY primed T cells were cultured in Experiments 1 and 2 and 2.5×10^6 HCY primed T cells were cultured in Experiment 3. HCY primed cells were taken 6 days after priming. DNP-HCY was at 2.5 µg/ml.

b) Cultures were assayed on day 4. Each value is the arithmetic mean of 4 wells ± the standard error of the mean.

c) Not done.

d) See also Table 5.3.

Table 3.10: Collaboration between DNP primed and HCY primed spleen cells in vivo

Cells transferred a)	Indirect anti-DNP PFC per spleen b)	
	Expt. 1	Expt. 2
DNP primed alone	1400 ± 440	1480 ± 260
HCY primed alone	2040 ± 420	n.d.
DNP primed + HCY primed	11100 ± 2100	19300 ± 4480
DNP primed + unprimed spleen	n.d.	900 ± 320

a) In experiment 1, 2×10^7 DNP primed and 5×10^7 HCY primed cells (6 days after priming) were transferred. In experiment 2, 2×10^7 DNP primed and 1.5×10^8 HCY primed cells (6 days after priming) were transferred. 100 µg DNP-HCY was given to each mouse.

b) Spleens were assayed on day 7 after cell transfer.

Table 3.11: Collaboration between DNP primed and HGG primed spleen cells in the anti-DNP response to DNP-HGG in vitro.

Cells cultured [a]	Anti-DNP PFC per culture [b]
DNP primed cells alone	20 ± 5
HGG primed cells alone	108 ± 12
DNP primed + HGG primed cells	351 ± 90
DNP primed + HCY primed cells	194 ± 22

a) 2×10^6 DNP primed and 5×10^6 carrier primed cells (6 days post priming) were cultured with 100 ng/ml DNP-HGG.

b) PFC were assayed on day 4.

IgM response, which peaked on day 3 resulted whether the mice had been deliberately primed beforehand or not (Table 3.12). To see, therefore, if the response to DNP-MON was merely a B cell effect without T cell involvement, spleen cells from nude mice, their littermates, ATxBM mice, normal CBA/H mice and deliberately primed CBA/H mice, were cultured with DNP-MON. The PFC response on day 3 is shown in Table 3.13. It can be seen that ATxBM mice and nude mice responded to this antigen in vitro as well as mice possessing T cells. There was also little indication that deliberate carrier priming could augment this response.

A. Nature of the antigen

The results in Tables 3.12 and 3.13 seemed contradictory to reports (e.g. Feldmann, 1972b; Feldmann and Basten, 1971) that the response to DNP-MON was dependent on T cells. Only the primed groups of mice were expected to respond. The antigen being used was further examined in some detail because of this discrepancy.

Dose effect or mitogenic properties

Spleen cells from nude mice, CBA/H and CBA/J mice were cultured with unconjugated MON or DNP-MON at concentrations ranging from 10-1000 ng/ml. The anti-DNP response is shown in Table 3.14. Nude mice gave as good a response as CBA/J mice over this range of antigen concentrations. The response of CBA/H mice, from our colonies, however, was about twice that of the nudes or CBA/J mice. Although there was some mitogenic effect of this MON preparation (unpurified) it did not account for the large response shown by nudes to DNP-MON.

In addition it was shown (Table 3.15) that the DNP had to be chemically coupled to the MON (a necessary requirement in classic hapten-carrier systems (Mitchison et al., 1970). The MON was not simply giving "signal 2" to a DNP-stimulated B cell (Bretscher and Cohn, 1970; Bretscher, 1972).

The effect of having both MON and DNP-MON in the cultures caused inhibition rather than enhancement of the anti-DNP response (Table 3.16).

Table 3.12: In vitro anti-DNP response to DNP-MON

Cells cultured [a]	Number per culture	Anti-DNP PFC per culture		
		Day 3	Day 4	Day 5
DNP primed alone	2×10^6	794 ± 52	630 ± 99	350 ± 64
DNP primed + MON primed	2×10^6 1×10^6	1968 ± 254	1030 ± 56	335 ± 36
DNP primed + MON primed	$.2 \times 10^6$ 2×10^6	2376 ± 138	1920 ± 179	440 ± 60
DNP primed + MON primed	2×10^6 4×10^6	3700 ± 674	2230 ± 332	588 ± 72
DNP primed + unprimed	2×10^6 2×10^6	2308 ± 342	1245 ± 96	n.d. [b]
DNP primed + unprimed	2×10^6 4×10^6	3632 ± 164	2140 ± 184	1068 ± 85

a) Cells were cultured with DNP-MON at a final concentration of 2 µg/ml.

b) Not done.

Table 3.13: Response of CBA/H and T deficient mouse spleen cells to DNP-MON in vitro

Cells cultured [a]	Anti-DNP PFC per culture [b]
Nu/nu	1650 ± 116
Nu/+	1485 ± 199
ATxBM	1003 ± 48
CBA/H	1785 ± 121
MON primed CBA/H	2265 ± 142
DNP primed + MON primed CBA/H	1700 ± 138

a) Unprimed cells were cultured at a concentration of 6×10^6 per well.

2×10^6 DNP primed cells were cultured with 4×10^6 MON primed cells.

DNP-MON was at a final concentration of 1 µg/ml.

b) Cultures were assayed on day 3.

Each value is the arithmetic mean of 4 wells ± the standard error of the mean.

Table 3.14: Effect of antigen dose and MON alone on the anti-DNP response

Cells cultured [a]	Antigen	Anti-DNP PFC per culture at different concentrations of antigen [b]			
		Nil	10ng/ml	100ng/ml	1000ng/ml
Nu/nu	-	40 ± 12			
	MON		75 ± 31	175 ± 47	394 ± 109
	DNP-MON		1150 ± 189	2069 ± 51	1600 ± 223
	-	-			
CBA/J	MON		88 ± 26	163 ± 26	294 ± 36
	DNP-MON		938 ± 122	1825 ± 68	1806 ± 139
	-	64 ± 10			
CBA/H	MON		656 ± 57	581 ± 60	956 ± 30
	DNP-MON		3363 ± 238	4888 ± 361	2769 ± 356

a) 5×10^6 cells per well.

b) Cultures were assayed on day 3.
Each value is the arithmetic mean of 4 wells ± the standard error of the mean.

Table 3.15 : The requirement for chemical union of the hapten and carrier

Antigen	Concentration (ng/ml)[b]	Direct anti-DNP PFC per culture [a]
-	-	130 ± 25
MON 870	100	150 ± 26
MON 870	1000	130 ± 17
DNP-HCY	2000	115 ± 44
DNP-HCY + MON 870	2000 100	140 ± 8
DNP-MON	100	1065 ± 128

a) Each value represents the arithmetic mean of four cultures ± the standard error of the mean (5×10^6 CBA/H spleen cells per well).

b) DNP-HCY was used at a concentration of 2 µg/ml as this had been found optimal for the response of CBA/H spleen cells to this antigen *in vitro*. (3.3.2.1.B).

Table 3.16: Effect of excess MON on the *in vitro* anti-DNP response to DNP-MON

Antigen concentration (ng/ml)		Anti-DNP PFC per culture a)b)	
DNP-MON	MON	Expt. 1	Expt. 2
100	-	1065 ± 128	1370 ± 50
100	100	530 ± 55	905 ± 152
100	1000	355 ± 48	953 ± 78

a) 5×10^6 cells were cultured per well.

b) Cultures were assayed on day 3. Each value is the arithmetic mean of 4 wells ± the standard error of the mean.

Degree of substitution of MON with DNP

Highly substituted hapten-protein conjugates have been reported to be able to yield T independent anti-hapten responses (Aird, 1971), presumably by presenting an array of repeating antigenic determinants to the B cell and activating it directly (Feldmann and Basten, 1971). The effect of the degree of substitution of the MON with DNP, on the response of nude and normal mice in vitro was investigated (Figure 3.3). At very low coupling ratios, nudes still gave a significant response which was again about half that of CBA/H mice when equal numbers of spleen cells were compared.

Possibility of contaminating DNP-POL

In most of the experiments reported so far the antigen was a MON preparation oxidized with chloramine-T before dinitrophenylation. This oxidation procedure has been shown to prevent repolymerization of the MON to POL (Parish and Stanley, 1972). However, although the response of DNP-MON is reported to be dependent on T cells, DNP-POL is able to induce a T cell independent anti-DNP response (Feldmann, 1972a). The possibility, therefore, that contaminating amounts of DNP-POL were responsible for the observed anti-DNP response was investigated.

Samples of both oxidized and non-oxidized DNP-MON preparations were centrifuged on sucrose gradients (18 h at 39500 rpm, 5^{o}C). Fractions were analyzed for protein concentration spectrophotometrically. Comparable gradients containing markers of known molecular weight were run so that the position of MON and aggregates of MON could be predicted. Only one protein peak was observed, coinciding with the monomeric protein, (although the possibility of contamination by dimers cannot be excluded) (Figure 3.4).

At least 80% of the total absorbance units applied were recovered in this peak in both the oxidized and non-oxidized preparations. Some labelled protein, presumably DNP-polymerized flagellin was sedimented at the bottom of the tube. The purified DNP-MON was used immediately as the antigen in tissue culture and compared with the original non-centrifuged preparations (Table 3.17).

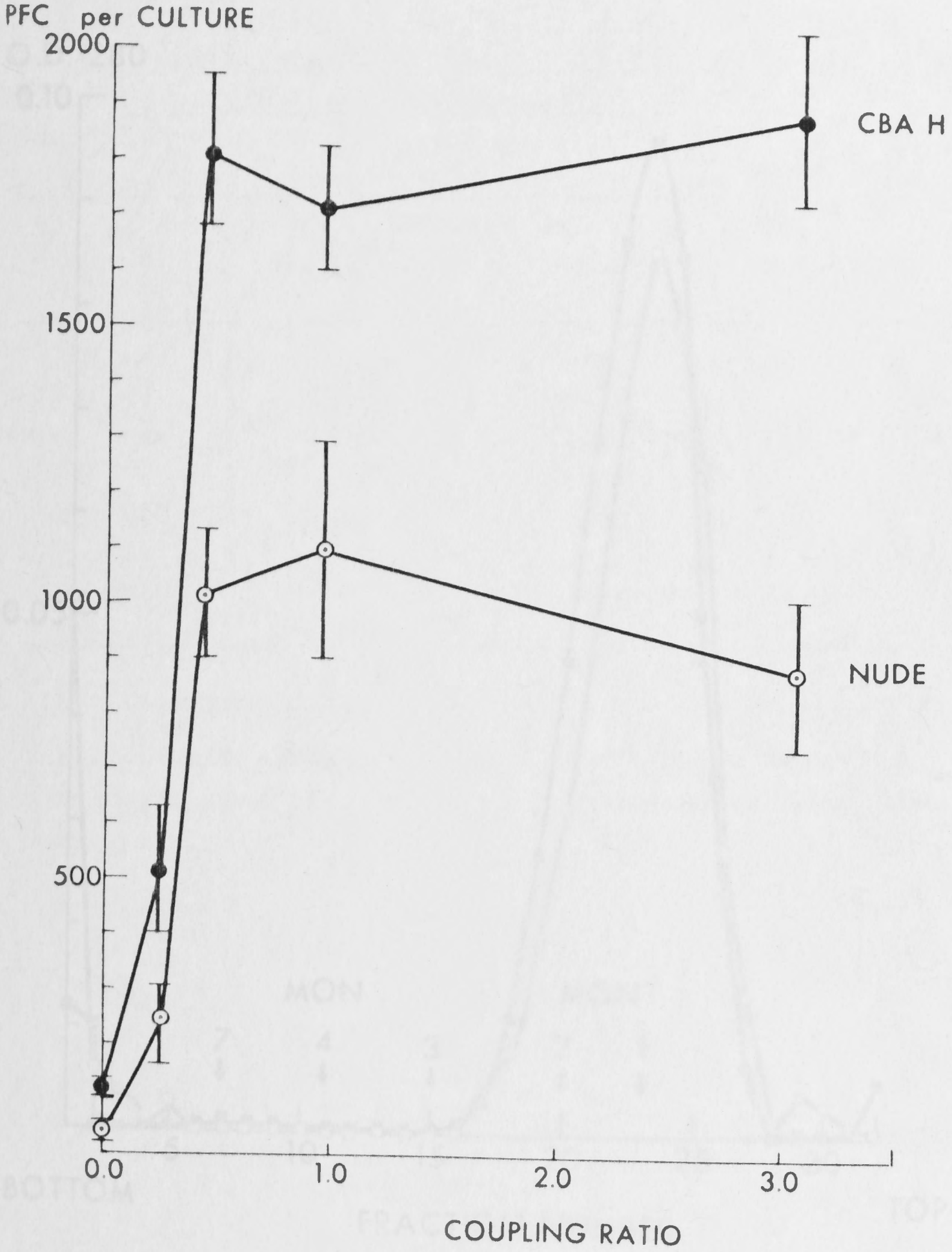


Figure 3.3 The effect of the degree of substitution of MON with DNP on the anti-DNP response of nude and normal CBA/H mice (5×10^6 cells/well). Each point represents the mean of 4 wells ± the standard error of the mean. The antigen was at a final concentration of 100 ng/ml in all cases.

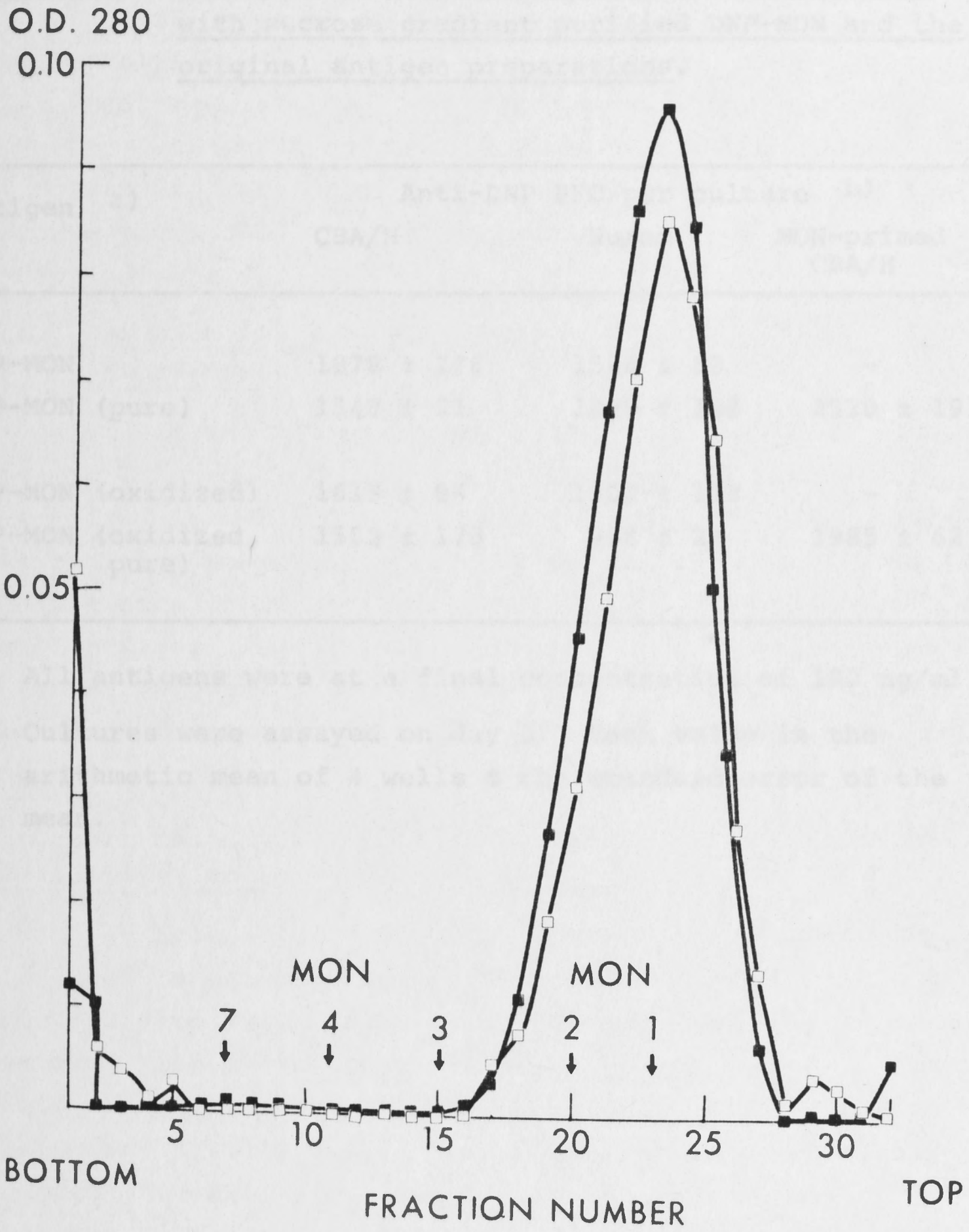


Figure 3.4 The protein profile obtained when oxidized ■- ■ and non-oxidized □- □ DNP-MON preparations were spun on sucrose gradients. A single protein peak corresponding to the monomer was obtained.

Table 3.17: Comparison of the anti-DNP response obtained with sucrose gradient purified DNP-MON and the original antigen preparations.

Antigen [a]	Anti-DNP PFC per culture [b]		
	CBA/H	Nu/nu	MON-primed CBA/H
DNP-MON	1878 ± 216	1548 ± 93	-
DNP-MON (pure)	1548 ± 21	1000 ± 132	2520 ± 191
DNP-MON (oxidized)	1613 ± 86	1503 ± 173	-
DNP-MON (oxidized, pure)	1503 ± 173	938 ± 25	1985 ± 62

a) All antigens were at a final concentration of 100 ng/ml.

b) Cultures were assayed on day 3. Each value is the arithmetic mean of 4 wells ± the standard error of the mean.

The results confirmed that both nude mice and normal "unprimed" mice gave good responses to DNP-MON in tissue culture, although there had been some contribution by DNP-POL in the nude response. There was only a slight effect of deliberate carrier priming of CBA/H mice. The purified DNP-MON was stored at a low concentration (10 µg/ml) for subsequent use.

B. Evidence for the importance of T cells in the response to DNP-MON

It was now seriously considered that nude mice might possess functional T cells, and that mice in our colonies were pre-primed to MON. In this next section the T-dependence of the anti-DNP response to DNP-MON is clearly established and the presence of MON primed T cells confirmed by a second assay for functional T cells, delayed-type hypersensitivity to MON injected into the hind-footpad. In the following chapter the presence of these functionally active T cells in nude mice is shown.

Effect of anti-θ and complement treatment on the response of CBA/H spleen cells to DNP-MON in vitro

Spleen cells from CBA/H mice were treated with anti-θ and complement and then cultured with DNP-MON. This treatment was cytotoxic for 20 to 30% of CBA spleen cells when compared with cells treated similarly with normal AKR ascitic fluid and complement. The PFC responses of such treated cells are shown in Table 3.18. Anti-θ treatment significantly reduced the ability of spleen cells to give an anti-DNP response. If the anti-θ ascitic fluid was adsorbed with CBA mouse brain (4 brains/ml) prior to use, the cytotoxicity for normal spleen cells was reduced from 25% to 5%, and this effect was paralleled in the tissue culture responses (Table 3.18). In addition, when DNP immune spleen cells were treated with the anti-θ and complement just prior to assay, no effect on the PFC response was observed.

Restoration of the anti-DNP response of anti-θ treated spleen by the addition of T cells

The importance of T cells in the response was confirmed by their ability to restore the anti-DNP response to

Table 3.18: The effect of anti-θ treatment on the anti-DNP PFC response

Treatment of cells [a]	Anti-DNP PFC per culture [b]				
	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5
Untreated	1193 ± 117	1303 ± 87	1860 ± 194	1275 ± 78	1073 ± 22
Normal ascitic fluid and C'	1185 ± 41	1120 ± 68	1403 ± 96 [d]	1233 ± 39	995 ± 10
Anti-θ ascitic fluid and C'	365 ± 39	390 ± 48	495 ± 91	217 ± 35	150 ± 23
Anti-θ (adsorbed) [c] and C'	n.d. [e]	975 ± 40	1068 ± 75	n.d.	n.d.

a) In all cases 5×10^6 viable cells were cultured per well with purified, oxidised DNP-MON at a final concentration of 100 ng/ml.

b) Each value is the arithmetic mean of 4 cultures ± the standard error of the mean.

c) The anti-θ ascitic fluid was adsorbed for 1 h at 0°C on CBA/H mouse brain slurry (4 brains/ml fluid).

c) The decrease in the anti-DNP response following treatment with normal ascitic fluid and C' was not due to the ascitic fluid but was a property of the batch of C' used.

e) Not done.

anti-θ treated spleen cells. Thymus cells could partially restore the response (Table 3.19). On a cell for cell basis, cortisone resistant cells gave better restorations than thymus cells, but again only partial restorations of the response were obtained (Table 3.19). If, however, the cortisone resistant thymus cells were cultured with MON for 1-2 days prior to their addition to the anti-θ treated spleen cells, they were able to restore completely the anti-DNP response (Table 3.20). It was shown by radioautography and the nigrosin staining procedure (2.2.11) that the cortisone resistant cells which proliferated and formed blasts during culture alone with MON were T cells, as they were killed by anti-θ serum and complement but not by anti-B cell serum and complement (2.3.2.4) (see Table 3.21 and Figure 3.5).

It will be shown (5.3.2.1) that splenic T cells, isolated on Isopaque/Ficoll, could also fully restore the anti-DNP response to anti-θ treated spleen cells. Hence the response to DNP-MON was dependent on T cells primed to MON and these were present in normal (i.e. not deliberately primed) spleen cells from mice in our colonies.

Collaboration between MON primed T cells and B cells in an anti-hapten response could also be demonstrated *in vivo*. See Table 5.7 and 7.6 and 7.7.

DTH responses in CBA/H mice

The presence of MON primed T cells in mice from our colonies was further substantiated by the ability of these mice to give DTH reactions to MON injected into the footpad (Cooper, 1972a). Similar responses were obtained when MON was injected into the hind footpad whether the mice had been deliberately primed with MON or not (Table 3.22 and Figure 3.6). The DTH response to HCY was negligible, however, unless the mice were deliberately primed, when equally large footpad swellings were registered.

3.4 DISCUSSION

This chapter has described the characteristics of the *in vitro* anti-hapten responses to DNP-HCY, DNP-HGG and

Table 3.19: Restoration of the anti-DNP PFC response of anti-θ treated spleen cells by the addition of thymus and cortisone resistant thymus cells

Cells cultured per well			Anti-DNP PFC per culture c)	
Spleen a)	Thymus b)	CRT b)	Expt. 1	Expt. 2
Untreated	- d)	-	1423 ± 79	1650 ± 31
Anti-θ and C' treated	-	-	285 ± 22	438 ± 35
Anti-θ and C' treated	2×10^6	-	503 ± 19	n.d. e)
Anti-θ and C' treated	4×10^6	-	655 ± 18	825 ± 64
Anti-θ and C' treated	8×10^6	-	678 ± 33	n.d.
-	4×10^6	-	0	0
Anti-θ and C' treated	-	3×10^6	835 ± 74	950 ± 54
-	-	3×10^6	<5	<5

a) 5×10^6 viable cells per well. DNP-MON at 100 ng/ml.

b) Thymus cells and cortisone resistant thymus cells (CRT) were from normal (i.e. not deliberately primed) CBA/H mice.

c) Cultures were assayed on day 3. Each value is the arithmetic mean of 4 wells ± the standard error of the mean.

d) No cells.

e) Not done.

Table 3.20: Restoration of the anti-DNP PFC response of anti-θ treated spleen cells by the addition of activated thymus cells

Cells cultured per well		Anti-DNP PFC per culture	
Spleen [a]	Activated thymus cells [b]	Expt. 1	Expt. 2 [c]
Untreated	-	1435 ± 64	1275 ± 78
Normal ascitic fluid and C'	-	1315 ± 134	1233 ± 39
Anti-θ ascitic fluid and C'	-	238 ± 26	217 ± 35
Anti-θ ascitic fluid and C'	1.5×10^6	1048 ± 138	780 ± 68
Anti-θ ascitic fluid and C'	3×10^6	1280 ± 198	1193 ± 107
Anti-θ ascitic fluid and C'	5×10^6	1403 ± 73	1185 ± 122
Anti-θ ascitic fluid and C'	7.5×10^6	1128 ± 71	1123 ± 60

a) 5×10^6 viable cells per well.

b) Cortisone resistant thymus cells were cultured for 2 days with MON, harvested, washed, counted and cultured as above for a further 3 days. DNP-MON was at a final concentration of 100 ng/ml.

c) Each value is the arithmetic mean of 4 wells ± the standard error of the mean.

Table 3.21: The effect of anti-θ and anti-B cell serum and complement on proliferating cortisone resistant thymocytes

Treatment of cells	Number of cells counted			
	Labelled		Unlabelled	
	Live	Dead[b)]	Live	Dead
Untreated	65	0	221	137
Normal rabbit serum and C'	42	0	159	111
Anti-B cell serum and C'	31	1	50	172
Anti-θ and C'	5	81	25	245

a) ^{3}HTdr uptake. See Figure 3.5.

b) As judged by the nigrosin staining procedure (2.2.11).

Figure 3.5

Radioautographs of MON activated cortisone resistant thymocytes. $^{3}HTdr$ uptake.

a) Cells treated with normal rabbit serum and complement after culture.

b) Cells treated with anti-B cell serum and complement after culture.

c) Cells treated with anti-θ ascitic fluid and complement after culture.

The labelled cells are unaffected in a) and b) but killed in c). Dead cells are stained with nigrosin.
Scale in microns.

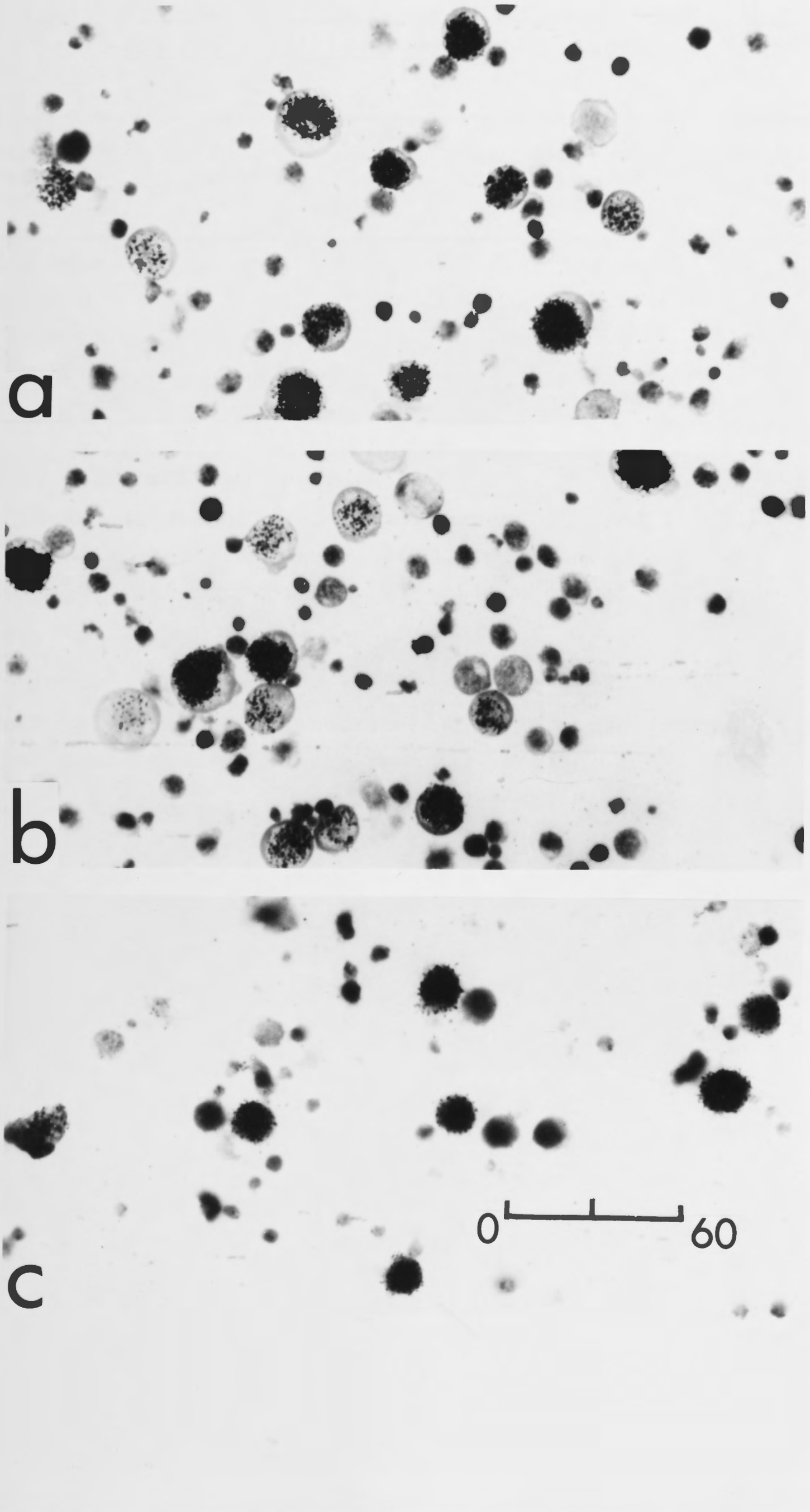

a
b
c
0
60

Table 3.22: Evidence for MON primed T cells in normal (i.e. not deliberately primed) CBA/H mice

Primary challenge [a]		Secondary challenge [b]	Mean percentage increase in footpad thickness at 24 hours [c]
CBA/H	None	MON	20.9 ± 1.6
	MON	MON	23.3 ± 2.7
	None	HCY	3.2 ± 1.0
	HCY	HCY	20.8 ± 3.0

a) HCY: 1 mg sub. cut. (flank) in 0.2 ml Freund's complete adjuvant (Difco) plus 1 mg aggregated HCY and 10^9 *B. pertussis* organisms, i.p., in 0.2 ml saline.
MON 870: 100 µg sub. cut. (flank) in 0.2 ml saline.

b) HCY: 1 mg, MON 870: 100 µg, sub. cut. (right hind footpad) in 0.01 ml saline, 6 days after primary challenge.

c) Each value is the arithmetic mean of 6 mouse footpads ± the standard error of the mean.

See also Figure 3.6.

Figure 3.6

Histological sections (haematoxylin eosin stained) through CBA/H mouse footpads 24 hours after the injection of antigen.

a) HCY into an unprimed mouse footpad.

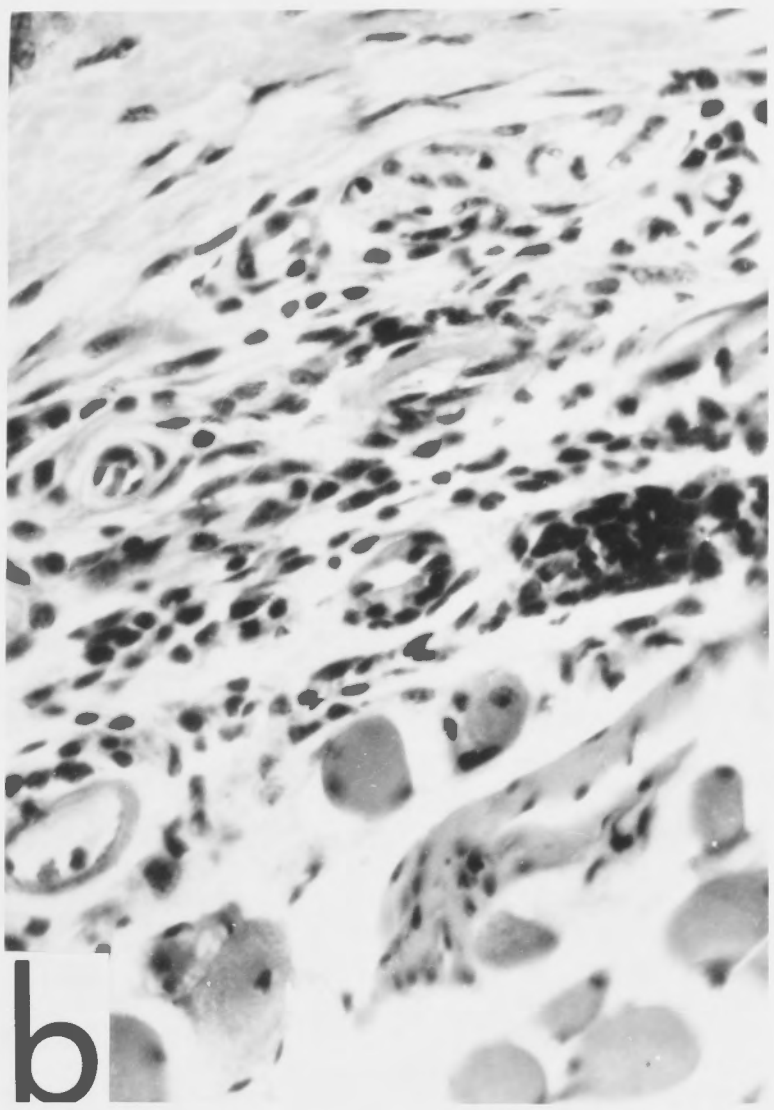
b) HCY into HCY primed mouse footpad.

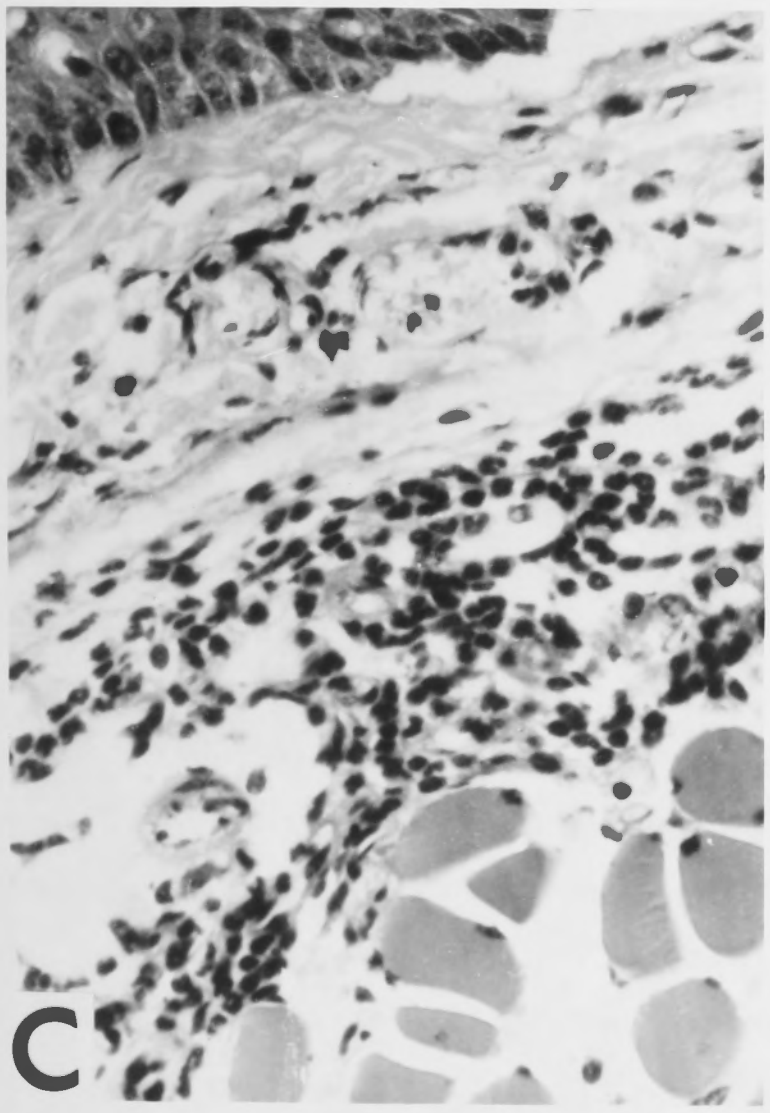
c) MON into an "unprimed" mouse footpad.

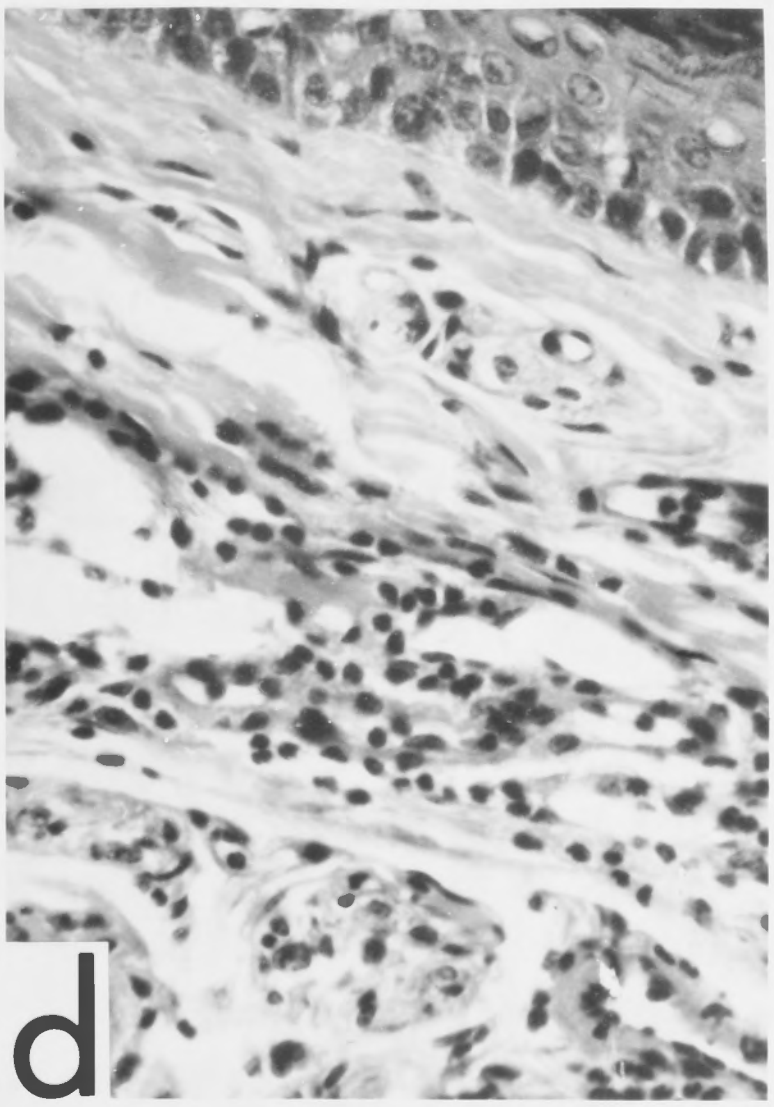
d) MON into a deliberately primed mouse footpad.

b), c) and d) show cellular infiltration and muscle oedema giving a DTH reaction (Magnification: approx. 300x).


a


b


c


d

DNP-MON. A PFC assay adapted from Strausbauch et al. (1970), was established and used for measuring anti-DNP PFC. The responses to DNP-HCY and DNP-MON were characterized in some detail. It was shown that for both antigens the anti-DNP response was dependent on collaboration between DNP specific B cells and carrier primed T cells. A role for the carrier primed B cells was also inferred from some preliminary experiments with the DNP-HCY system. Treatment of the HCY primed population with anti-Ig serum and complement severely interfered with its ability to help in the response to DNP-HCY. The role of the carrier primed B cells will be pursued in later chapters (Chapters 6 and 7).

The response to DNP-HCY *in vitro* was similar to anti-hapten responses reported previously *in vivo*, (Mitchison, 1970; Mitchison, 1971; Roelants and Askonas, 1971) - minimal responses to the antigen being obtained unless populations primed to both the carrier and the hapten were present. In this case a 2½ to 3 fold increase in the PFC response was obtained above background (i.e. the PFC response when hapten primed B cells were cultured with heterologously primed T cells). This difference appeared to be the limit obtainable in culture (3.3.2.1.B). Purified T cells (Isopaque/Ficoll procedure, Chapter 5) from the HCY primed population collaborated with the hapten primed population in the anti-DNP response. This represented a direct demonstration of T-B collaboration *in vitro*.

The *in vitro* response to DNP-MON was much more vigorous than the response to DNP-HCY. A larger PFC response (2-4 times greater), which arose more rapidly, (peak response day 3 compared to day 4 for DNP-HCY) was obtained even without deliberate carrier priming and even in mice severely depleted or supposedly free of T cells. Nevertheless, it was clearly established that this response represented a collaboration between carrier-primed T and hapten-primed B cells. The difference between this response and the one to DNP-HCY was that animals from our school had become naturally primed to flagellin or cross reacting antigens from birth and therefore had built up significant numbers of MON primed T cells. This was particularly striking in the response of ATxBM mice and

the congenitally athymic "nude" mouse mutant which could respond almost as well as normal mice to this T dependent antigen (see also Chapter 4).

Evidence that the response to DNP-MON was due to true T-B collaboration (as had been previously reported by Feldmann, 1972b) and not due to mitogenic properties of the antigen for B or T cells, can be summarized as follows:-

a) Purified MON alone in the culture at concentrations used, did not cause an anti-DNP response above background levels.
b) The same large response to DNP-MON was obtained with 5 different DNP-MON preparations.
c) Rigorous removal of DNP-POL from the preparations made little difference to the anti-DNP response.
d) Chemical linkage between the DNP and MON was required for an anti-DNP response. The MON was not simply giving a second signal to a DNP activated B cell.
e) Excess MON in the culture inhibited, rather than stimulated, the response to DNP-MON.
f) The response was dependent on the presence of primed T cells. Anti-θ treatment reduced it 3-7 fold. Thymus cells and cortisone resistant thymus cells could only partially restore it but MON activated cortisone resistant thymus cells and splenic T cells (Chapter 5) could fully restore it.

Evidence that the CBA/H mice were pre-primed to MON was:-

a) Deliberately priming the mice with MON led to no augmentation of the anti-DNP response. In addition, maximal DTH to MON was obtained without deliberate priming, in contrast to results of Cooper, (1972a) using similar mice from these colonies and the same antigen preparation.
b) Newborn mice (1-5 days after birth) did, however, show a 3-5 fold increase in the anti-DNP response to DNP-MON following deliberate MON priming. (I. Ramshaw, personal communication.) CBA/J mice purchased from overseas which had spent less time with our colonies, gave consistently lower responses than mice bred in the School. (3.3.2.3.A).

Thus two *in vitro* systems for measuring T cell helper function were characterized. The response to DNP-HCY has been used to investigate collaboration between histoincompatible T and B cells, reported elsewhere, (Zinkernagel, Kirov and Doherty, in preparation), and in characterizing the Isopaque/Ficoll separation procedure (Chapter 5). The response to DNP-MON has proved more useful due to its extreme sensitivity to T cells. It has been used in some collaborative work with Dr. A. Globerson to investigate whether uncommitted stem cells in embryonic liver have the capacity to develop into T helper cells on passage through the thymus (Kirov and Globerson, 1974), in characterizing the Isopaque/Ficoll separation (Chapter 5), and in the experiments described in Chapters 6 and 7 where it was used in conjunction with the cell separation procedure to look at the mechanisms of collaboration between T and B cells.

3.5 SUMMARY

In vitro culture systems for measuring the anti-hapten responses to DNP-HCY and DNP-MON were set up and characterized. Specific anti-DNP PFC were measured using SRBC coated with rabbit anti-sheep Fab-DNP reagent. The response to both hapten-carrier antigens was shown to be dependent on the presence of carrier primed T cells and this was demonstrated directly for both systems. The extremely good response obtained to DNP-MON was shown to be due to natural pre-priming of the animals to the carrier determinant or a cross reacting antigen. A possible role for carrier primed B cells in collaboration was also intimated by the ability of anti-Ig and complement treatment of carrier primed spleen cells to interfere with the anti-hapten response to DNP-HCY.

CHAPTER 4

Demonstration of functionally active T cells in congenitally athymic ("nude") mice

4.1 INTRODUCTION

The congenitally athymic ("nude") mouse mutant (Flanagan, 1966) lacks recognizable thymus tissue, at least in adult life, and seems to be severely depleted of thymus-derived lymphocytes by both histological and functional criteria. It is grossly depleted of lymphocytes in the thymus-dependent areas of lymph nodes and spleen (de Sousa et al., 1969), and is deficient in its ability to reject tissue grafts (Rygaard and Povlsen, 1969; Rygaard, 1969), to give a cell-mediated cytotoxic response *in vitro* (Feldmann et al., 1972), to produce antibodies to "thymus-dependent" antigens, e.g. SRBC and T_4 phage (Kindred, 1971; Wortis, 1971), and to react to T cell mitogens, e.g. PHA and Con A (Wortis, 1971; Coutinho et al., 1973). In addition, theta-positive lymphocytes have been reported few or missing (Raff and Wortis, 1970), and virtually 100% of nude thoracic duct lymphocytes has been found to have a B cell receptor for antigen-antibody complexes (Bankhurst and Warner, 1972).

However, recent reports (Loor and Kindred, 1973; Loor and Roelants, 1974) have demonstrated precursor T cells present in nude mice, detected by a fluorescent anti-θ reagent, which can repopulate a thymus graft. It has not been shown that nude mice possess functionally active T cells, but as reported in the previous chapter (3.3.2.3) nude mice from our colonies gave extremely good responses to DNP-MON *in vitro*. Responses to this antigen were shown to be dependent on the presence of MON primed T cells (3.3.2.3.B) and in this chapter evidence is presented that nude mice do in fact have a small percentage of theta-positive lymphocytes which are capable of functioning as specific "helper" cells, and which are able to mount a delayed type hypersensitivity reaction to flagellin injected into the hind footpad.

4.2 MATERIALS AND METHODS

4.2.1 Animals

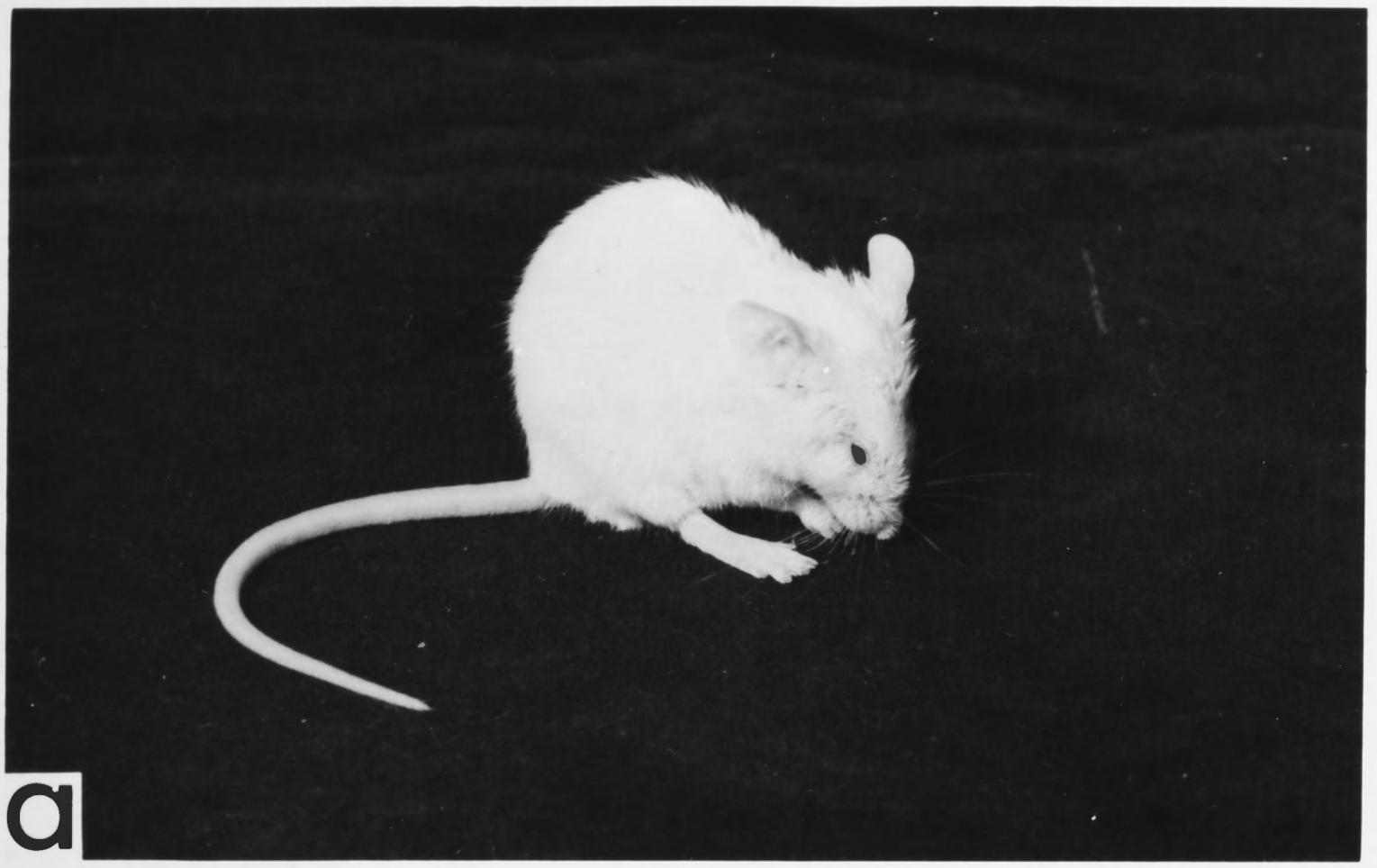
Mice used were obtained from breeding colonies in this School. Nude mice were from both heterozygous (nu+ x nu+) and homozygous (nu+ x nu/nu) matings of mice obtained

Figure 4.1

a) The "nude" mouse littermate (nu/+).

b) The congenitally athymic "nude" mouse mutant (nu/nu).

These two mice came from a homozygous mating of a nu/+ female with a nu/nu male.



a

b

originally from Dr. M. Holmes of the Walter and Eliza Hall Institute, Melbourne. They had no signs of thymus tissue. The heterozygous nudes, originally outbreds, had been back-crossed in CBA/H and Balb c mice. Mice were used at 7-9 weeks of age. (See Figure 4.1).

4.2.2 Culture conditions and assay procedures

Antigens, culture conditions, cell treatments and assay procedures have all been described in the previous chapter (3.2).

4.3 RESULTS

4.3.1 Ability of nude mice to respond to DNP-MON in vitro

As reported in Chapter 3 (3.3.2.3), both nude and normal mice gave good responses to DNP-MON without any deliberate priming. The response to other DNP conjugates was minimal, however, unless cell populations primed to both the carrier and DNP were used. In this case normal mice gave good anti-hapten responses. These findings are summarized in Table 4.1. It can be seen that nude mice responded to DNP-MON as well as mice possessing T cells. The response, as for CBA/H mice (Table 3.15) was a classical anti-hapten response, chemical linkage between the hapten and the carrier being required (Table 4.2).

4.3.2 Effect of treatment with anti-θ and complement on the response

In view of these results, the possibility that nude mice might contain a proportion of T cells primed to MON seemed likely. To investigate this, spleen cells from nude mice were treated with anti-θ and complement and then cultured with DNP-MON, as had been done for CBA/H spleen cells (3.3.2.3.B). This treatment was cytotoxic for 20 to 30% of CBA spleen cells but only a small proportion of nude spleen cells (estimated to be between 2-4%) were killed when compared with cells treated similarly with normal AKR ascitic fluid and complement. The PFC responses of such treated cells are shown in Table 4.3. Anti-θ treatment significantly reduced the ability of nude mice to give an anti-DNP response.

Table 4.1: Response of CBA/H and nude mice to DNP conjugates in vitro

Cells cultured [a, b]	Antigen	Anti-DNP PFC per culture [c]
CBA/H normal	DNP-MON	1746 ± 151
CBA/H MON primed	DNP-MON	2265 ± 142
CBA/H DNP + MON primed	DNP-MON	1700 ± 138
Nu/nu	DNP-MON	1650 ± 116
Nu/+	DNP-MON	1485 ± 199
CBA/H normal	DNP-HCY	93 ± 4
CBA/H DNP + HCY primed	DNP-HCY	819 ± 18
Nu/nu	DNP-HCY	19 ± 6
CBA/H normal	DNP-HGG	40 ± 8
CBA/H DNP + HGG primed	DNP-HGG	351 ± 38
Nu/nu	DNP-HGG	10 ± 5

a) Unprimed cells were cultured at a concentration of 6×10^6 cells per well.
Antigen concentrations: DNP-MON 1 µg/ml
DNP-HCY 2 µg/ml
DNP-HGG 2 µg/ml

b) 2×10^6 DNP primed cells were cultured with 4×10^6 carrier primed cells.

c) Each value represents the arithmetic mean of four wells ± the standard error of the mean.

Table 4.2: The requirement for chemical union between the hapten and carrier for nude mice to respond to DNP-MON

Antigen	Concentration[b]	Anti-DNP PFC per culture a)
MON	100 ng/ml	20 ± 20
MON	1000 ng/ml	20 ± 20
DNP-HCY	2 µg/ml	30 ± 30
DNP-HCY + MON	2 µg/ml 100 ng/ml	20 ± 20
DNP-HCY + MON	2 µg/ml 1000 ng/ml	10 ± 10
DNP-MON	100 ng/ml	840 ± 128
DNP-MON	1000 ng/ml	620 ± 54

a) Each value represents the arithmetic mean of four wells ± the standard error of the mean (5×10^6 nu/nu spleen cells per well).

b) DNP-HCY was used at a concentration of 2 µg/ml as this was found optimal for response of CBA/H mice *in vitro*. (3.3.2.1 B).

Table 4.3: The effect of anti-θ treatment on the ability of nude spleen to respond to DNP-MON in vitro

Treatment of cells a)	Anti-DNP PFC per culture b) Expt. 1	Expt. 2	Expt. 3
Untreated	(828 ± 109	1020 ± 28	1178 ± 117
Normal ascitic fluid and C' c)	(705 ± 55	913 ± 47	978 ± 42
Anti-θ ascitic fluid and C'	335 ± 39	310 ± 30	405 ± 28

a) In all cases 5×10^6 viable cells were cultured per well with purified, oxidised DNP-MON at a final concentration of 100 ng/ml.

b) Each value is the arithmetic mean of 4 cultures ± the standard error of the mean.

c) The decrease in the anti-DNP response following treatment with normal ascitic fluid and C' was not due to the ascitic fluid but was a property of the batch of C' used. Even after rigorous adsorption, some batches of C' had residual anti-thymocyte activity which became important when only few T cells were involved.

4.3.3 Fractionation of nude spleen on Isopaque/Ficoll - enrichment of functional nude T cells

The presence of functionally active T cells in nude spleen was unequivocally shown following fractionation of cells on Isopaque/Ficoll (Chapter 5). When nude spleen was depleted of Ig-rosette forming cells there was a 10% recovery of non-rosetting cells in the upper layer. Of these cells, 32% were sensitive to treatment with anti-θ and complement. There was thus more than a ten-fold enrichment of the theta-bearing population in the upper layer. These cells were able to restore fully the response of an anti-θ treated nude spleen (Table 4.4). In addition, the Ig-bearing cells recovered from the bottom layer, following lysis of the SRBC, gave little response when cultured alone, but a normal response following re-addition of cells from the upper layer. The upper layer cells when cultured alone, gave no plaques at all.

4.3.4 Ability of nude mice to give a DTH response to MON

An independent assay, the measurementof delayed type hypersensitivity (DTH) to MON injected into the footpads (see 3.2.19), confirmed the presence of MON primed T cells in nude mice. DTH responses to MON were obtained in nude mice and were of the same magnitude as those obtained in CBA/H mice (Table 3.22), whether the mice were deliberately primed with MON or not (Table 4.5). Nude mice deliberately primed to HCY, however, did not develop footpad swellings, unlike their hairy littermates or CBA/H mice (Figure 4.2.).

4.4 DISCUSSION

The results reported in this and the previous chapter lead to the conclusion that nude mice possess a small number of theta-positive cells, a proportion of which have become primed to MON and can function as helper cells in a collaborative anti-DNP response and give a vigorous delayed-type hypersensitivity reaction to flagellin injected into the footpad. This conclusion rests on the following premises:

a) The antigen used in the tissue culture was DNP-MON, which did not contain any DNP-POL. DNP-MON is known to require MON-primed T cells in order to stimulate

Table 4.4: Fractionation of functionally active T cells on Ficoll gradients

Cells cultured			Anti-DNP PFC per culture d)
Unfractionated Spleen a)	Bottom layer b) (B cells)	Top layer c) (T cells)	
Untreated	-	-	800 ± 80
Normal ascitic fluid and C'	-	-	558 ± 43
Anti-θ and C'	-	-	168 ± 19
Anti-θ and C'	-	+	603 ± 84
-	-	+	0
-	+	-	285 ± 24
-	+	+	791 ± 58

a) and b) 5×10^6 viable cells were cultured per well.

c) 1.5×10^6 viable cells were cultured per well. B cells = lower layer from Isopaque/Ficoll separation of Ig-bearing and θ-positive lymphocytes. T cells = upper layer from separation.

d) Each value is the arithmetic mean of four cultures ± the standard error of the mean. DNP-MON was at a final concentration of 100 ng/ml.

Table 4.5: The ability of nude mice to give a DTH response to MON without deliberate priming

Mice	Primary a) Challenge	Secondary b) Challenge	Mean percentage increase in footpad thickness at 24 hours c)
Nude (Nu/nu)	None	MON	29 ± 1
	MON	MON	28 ± 1
	None	HCY	0
	HCY	HCY	0
Nude littermate (Nu/+)	None	MON	29
	MON	MON	26 ± 2
	None	HCY	3.9 ± 0.75
	HCY	HCY	17.3 ± 1.7

a) HCY: 1 mg sub. cut. (flank) in 0.2 ml Freund's complete adjuvant (Difco) plus 1 mg aggregated HCY and 10^9 B. pertussis organisms, i.p., in 0.2 ml saline.
MON 870: 100 µg sub. cut. (flank) in 0.2 ml saline.

b) HCY: 1 mg, MON 870: 100 µg, sub. cut. (right hind footpad) in 0.01 ml saline, 6 days after primary challenge.

c) 3-4 mice per group.

Figure 4.2

Histological sections (haematoxylin eosin stained) through nude mice footpads 24 hours after the injection of antigen.

a) HCY into an unprimed mouse footpad.

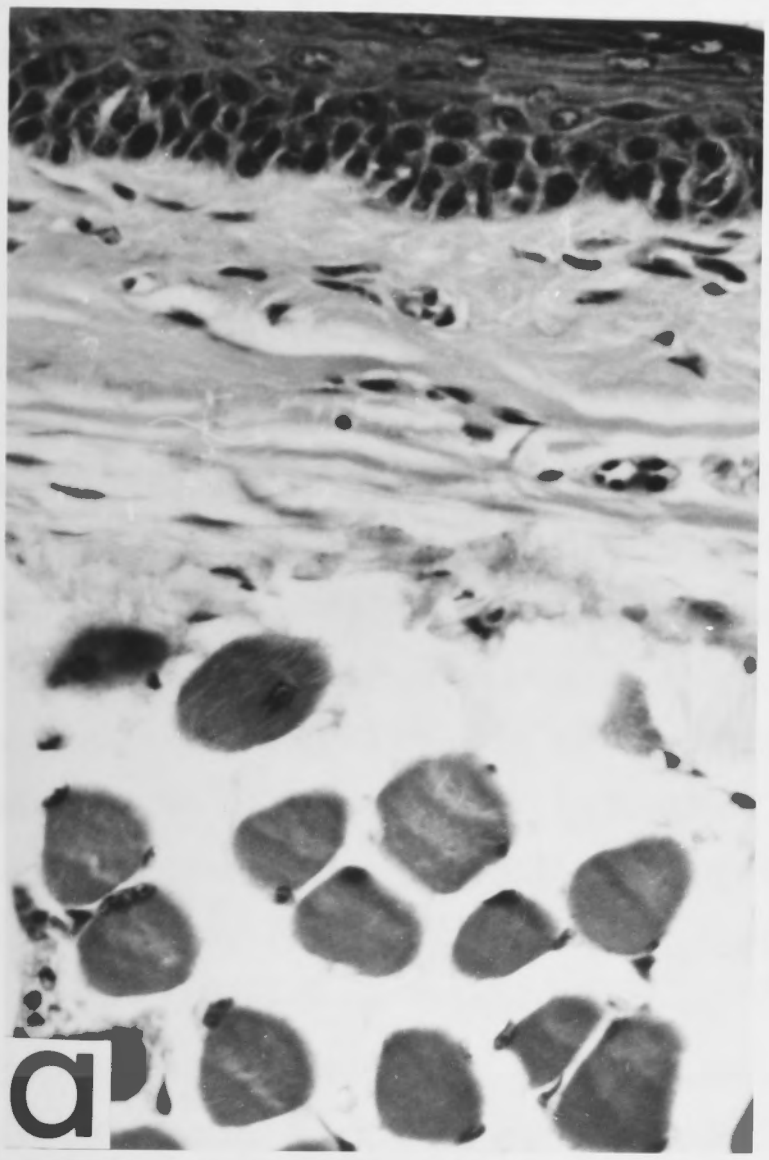
b) HCY into a HCY primed mouse footpad.

c) MON into an "unprimed" mouse footpad.

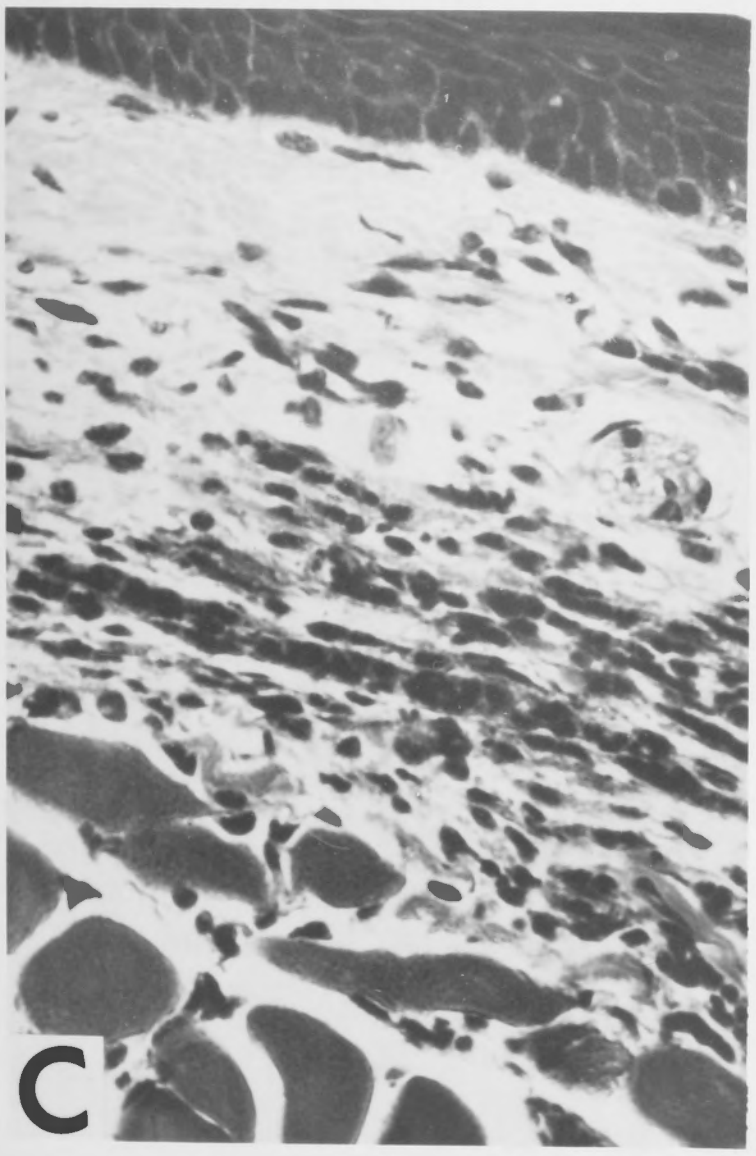
d) MON into a deliberately primed mouse footpad.

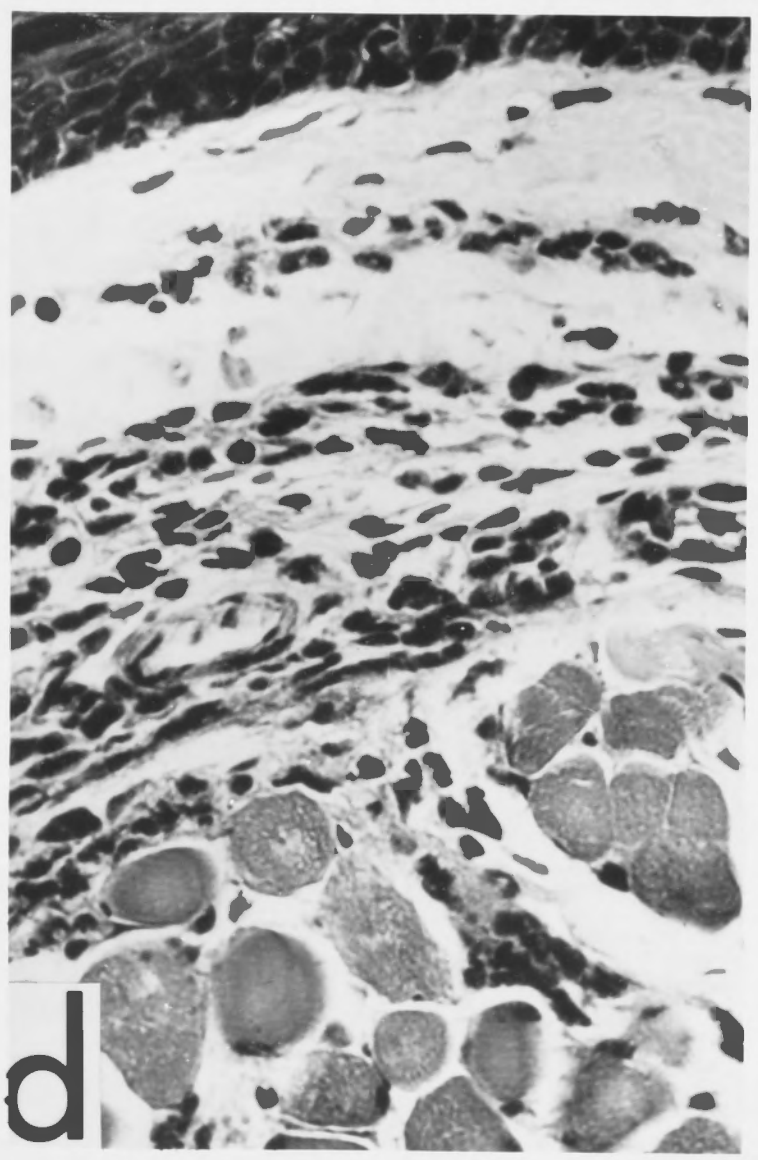
a) and b) show no DTH response.
c) and d) show massive cellular infiltration and muscle oedema giving a classic DTH reaction.

(Magnification: approx. 300x).


a


b


c


d

a good anti-DNP response.

b) Anti-θ treatment of spleen cells significantly reduced the anti-DNP response by specifically killing the T helper cells.

c) Mice in our colonies are pre-primed to the carrier, MON.

With respect to these premises, firstly, the unexpectedly high response of the nude mice to DNP-MON was not due to properties of the antigen used, e.g. its concentration in the cultures, non-specific mitogenic properties, high substitution ratios of the protein with DNP, or the presence of DNP-POL in the preparation (3.3.2.3.A). Secondly, the activity of the anti-θ used could a) be adsorbed by CBA brain tissue and b) did not affect anti-DNP PFC (3.3.2.3.B). In addition fractionation of nude spleen cells on Isopaque/Ficoll enabled confirmation of the importance of T cells in the response, showing that B cells alone could not respond and that addition of only a small number of T cells could completely restore the response of anti-θ treated spleen and of the T-depleted B population.

Evidence for c) was the finding that deliberate priming of mice in our colonies with MON had only a slight, if any, enhancing effect on two responses to this antigen. Thus, such mice were able to mount a vigorous DTH reaction on primary challenge with MON. This observation is consistent with the very slight effect of deliberate MON priming on the anti-DNP response (Tables 3.12, 3.13 and 4.1).

Mice may well be experiencing continual exposure to either flagellar or cross-reacting antigens so that a significant population of carrier-primed T cells is acquired naturally. It is of interest that mice of the same strain kept in this school did not exhibit this pre-priming two years ago (Cooper, 1972a). This suggests that the priming in mice may be due to an increased susceptibility to a particular bacterial infection.

These results, therefore, show that for functional tests nude mice, although depleted of T cells, cannot always be regarded as a source of uncontaminated B cells. The embryonic nude mouse had thymic epithelium at 13-14 days, which has disappeared by birth (M. Moore and A. Szenberg, personal communication). It is probable that the progenitors

of the functional T cells found in nude mice arose from this rudimentary thymus.

In addition, these results may be relevant to the findings of Morris and colleagues (1971, 1973) who reported that lambs thymectomized during foetal life gave undiminished humoral responses to three antigens, swine influenza virus, chicken red blood cells and Salmonella muenchen. It was concluded that in sheep, T cells were not required to give normal humoral responses to antigens. However, it is known (Cheng and Trentin, 1967) that in mice the background level of splenic PFC to SRBC is influenzed by the levels of certain bacteria in the gastrointestinal tract. If these three antigens used by Morris et al. can be regarded either as environmental antigens or related to environmental antigens, it seems very likely that at the time of thymectomy, sufficient T cells may have already been circulating to serve as a progenitor population of primed T cells for these antigens. After all, thymectomy cannot be performed unless a visible thymus is present. This proposal could be checked if a marker similar to theta antigen were available to distinguish B and T cells in sheep.

4.5 SUMMARY

Spleen cells from congenitally athymic ("nude") mice were found to give a good anti-hapten response when cultured with the hapten-carrier, DNP-flagellin, but not when cultured with DNP coupled to two other proteins, haemocyanin and human gamma globulin. This response to DNP-flagellin was anti-theta-sensitive. The evidence indicated that the mice had a small population of T cells, a proportion of which had become primed naturally to flagellin or to related antigens and could function as specific helper cells in a collaborative anti-DNP response. The existence of flagellin-primed T cells was confirmed by the ability of such mice to mount a DTH reaction to flagellin injected into the hind footpad.

CHAPTER 5

Characterization of a one step procedure for separating mouse T and B lymphocytes

5.1 INTRODUCTION

In this chapter a simple, one-step procedure is described for separating mouse T and B lymphocytes. A similar procedure had been worked out by Parish and Hayward (1974 a, b) for the rat and, in collaboration with Dr. Parish, was readily adapted to the mouse situation. The efficiency of the separation and the functional characteristics of the separated cell populations in the anti-hapten response to DNP-HCY and DNP-MON (Chapter 3) were looked at. The procedure was also adapted for separating and examining Fc receptor lymphocytes (FcRL).

5.2 MATERIALS AND METHODS

5.2.1 Animals, antigens and tissue culture procedures

As described in Chapter 3.

5.2.2 Radioautographic procedure

As described in Chapter 2.

5.2.3 Antisera

Antisera to mouse IgG and rabbit IgG were prepared by Dr. M.J. Crumpton and Dr. C.R. Parish. Briefly, mouse IgG and rabbit IgG were obtained from an $(NH_4)_2\ SO_4$ precipitation (40% saturation) of mouse or rabbit serum respectively. After extensive washing the precipitate was dissolved in a 20mM sodium phosphate buffer, pH 8.0, and passed through a DEAE column eluted with the same buffer. The protein eluted was then concentrated by pressure dialysis, applied to a Sephadex G-200 column, and the IgG fraction from the column collected and concentrated.

Antisera to rabbit Ig were raised in outbred merino sheep by injecting into multiple sites 1 mg of rabbit IgG emulsified in FCA (1.0 ml/sheep of a 1:1 mixture of FCA and antigen). Four to six weeks later the animals were boosted with 1mg of rabbit IgG in FIA and the sheep were bled out 7-14 days after the secondary challenge. The globulin fraction of this antiserum was precipitated with $(NH_4)_2SO_4$ at 40% saturation and after extensive washing the precipitate was redissolved in 0.15 M NaCl.

Antisera to mouse Ig were raised in rabbits by an identical immunization schedule.

Anti-SRBC antisera were raised in mice by subcutaneous injection of 10% SRBC in FCA (1:1 mix). Fourteen days later the mice were challenged with 0.5 ml 2% SRBC intraperitoneally and 7 days later they were bled and sera collected. The sera was inactivated (56^{o}C, 30 min) and stored in separate ampoules at -70^{o}C. A new ampoule was used for each experiment as the activity of the sera was decreased by freeze-thawing.

5.2.4 Rosetting procedure

5.2.4.1 Surface Ig rosetting

Mouse lymphocytes were rosetted for surface immunoglobulin by modifying a procedure previously developed for rosetting Ig-bearing rat lymphocytes (Parish and Hayward, 1974b). The procedure consisted of two stages; first, reaction of mouse lymphocytes with rabbit anti-mouse Ig and second, rosetting of the lymphocytes with SRBC coated with sheep IgG specific for rabbit Ig.

To 2.5ml of spleen or lymph node cells (4×10^7 cells/ml) in PBS/10% FCS was added 10µl of a hyperimmune rabbit anti-mouse Ig antiserum. In preliminary experiments this antiserum had been titrated against spleen cells and a dilution selected at which the number of Ig-rosetting cells had reached a plateau level. The cell-antiserum mixture was incubated on ice for 30 min and then centrifuged (300g, 5 min at 4^{o}C). The lymphocyte pellet was resuspended in 2.5ml of PBS/10% FCS and 2.5ml of a 20% suspension of SRBC (2×10^9 SRBC/ml) coated, via $CrCl_3$, with sheep IgG specific for rabbit Ig added (i.e., E anti-Ig). The method of coating SRBC with IgG preparations has been fully described by Parish and Hayward (1974b). In most experiments a control preparation was included where 2.5ml of a 20% suspension of unsensitized SRBC (E) was added to 2.5ml of lymphocytes. The erythrocyte-lymphocyte mixtures were then centrifuged (5 min, 1200g, 4^{o}C) to enable rosette formation. The cell pellets were gently resuspended in their supernatants and samples taken, diluted and examined for rosette content.

5.2.4.2 Fc rosetting

Equal volumes of a 5% suspension of washed SRBC (E) in PBS and the optimal dilution of mouse anti-SRBC antibody (A) in PBS were incubated at 37°C for 30 min. The sensitized cells were washed three times (300g, 5 min) with PBS, and the EA pellet resuspended by vortexing after each centrifuging in order to minimize red cell aggregation. After washing, a 20% suspension of EA in F15 culture medium was prepared and stored on ice. Rosettes were formed with lymphocytes as for 5.2.4.1.

Rosette-forming cells were counted as follows. To 0.1ml of the rosetted sample (2×10^6 lymphocytes/ml, 1×10^8 red cells/ml) was added 0.1 ml of PBS containing 1% (v/v) glutaraldehyde and 2% (w/v) crystal violet. This staining solution was clarified of undissolved dye by filtration prior to use. With crystal violet the white cells stained violet and could be distinguished whether free or binding red cells. Thus, this procedure guarded against aggregates of red cells being mistaken for rosettes. Preparations were counted immediately after addition of the staining solution as the violet colour of the white cells faded in 5-10 min. Approximately 300 white cells were scanned from each suspension for rosettes. The results were expressed as the percentage of rosettes in relation to the total number of nucleated cells. Any lymphocyte which bound >5 erythrocytes was classified as a rosette.

5.2.5 Separation of rosetting and non-rosetting cells

Spleen cells were rosetted for Ig-bearing or Fc receptor bearing cells as described above. To 5.0ml of rosetted lymphocytes (2×10^7 white cells/ml, 1×10^9 red cells/ml) was added 20 µl 25% (w/v) sodium azide (final concentration 0.1%) and the temperature of the mixture raised to 20°C in a water bath. The preparation was then layered gently on 4.0ml of separating medium which had been prewarmed to 20°C and which consisted of 12 parts of 14% (w/v) Ficoll (Pharmacia, Uppsala, Sweden) dissolved in distilled water and 5 parts of 32.8% (w/v) sodium metrizoate (Isopaque; Nyegaard and Co., Oslo, Norway), the complete mixture containing 1.0%

(w/v) sodium azide. This mixture had a density of 1.09, was stored at 4°C protected from light, and will be referred to as Isopaque/Ficoll. It was sterilized by Millipore filtration.

The separations were carried out in 12ml, U-bottomed, polycarbonate centrifuge tubes (16 x 100mm; cat. no. 272; Ivan Sorvall Inc., Norwalk, Connecticut, U.S.A.). After addition of the rosetting mixtures the tubes were placed in a centrifuge prewarmed to 20°C and spun at 2000g (at the Isopaque/Ficoll interface) for 20 min. The best separations were obtained if the centrifuge accelerated rapidly and attained 2000g within 20 seconds. After centrifugation (see Figure 5.1) the supernatant above the Isopaque/Ficoll interface was discarded and the white cell layer at the interface, together with all the separating medium above the red cell pellet, collected. The white cell preparation ("upper layer") was then diluted with medium, mixed and the white cells pelleted by centrifugation (300g, 10 min, 4°C). The pelleted cells were washed once more and counted.

The red cells in the rosette-red cell pellet ("lower layer") were lysed by osmotic shock as previously described by Dain and Hall (1967). Briefly, each pellet (0.5ml packed SRBC) was quickly resuspended in 8.55 ml. of 40% Ringer's solution and after all cell clumps had been dispersed isotonicity was restored by the addition of 1.0ml of 940% Ringer's solution. The white cells were then pelleted by centrifugation (300g, 5 min, 4°C), washed once more and counted.

5.3 RESULTS

5.3.1 Efficiency of the Isopaque/Ficoll cell separation procedure

5.3.1.1 Partitioning of mouse spleen cells on Isopaque/Ficoll

Table 5.1 summarizes the partitioning of sub-populations of mouse spleen cells on Isopaque/Ficoll. It was found that virtually all red cells (99.7%) sedimented to the bottom of the tube whereas the bulk of the white cells floated at the Isopaque/Ficoll interface (Figure 5.1). Some white

Table 5.1: Partitioning of mouse spleen cells on Isopaque/Ficoll. [a]

Cell type	Upper layer	Lower Layer	Recovery
Erythrocytes	0.3%	99.7%	100%
Leucocytes	89(98)[b]	9(56)	98
- Mononuclear	86	10	96
- Polymorphonuclear	93	4	97
- Stem cells c)	99	1	100

a) In this experiment 1 x 10^8 mouse spleen cells were applied to the Isopaque/Ficoll.

b) Values in parentheses represent % viability of cell populations as assessed by trypan blue exclusion.

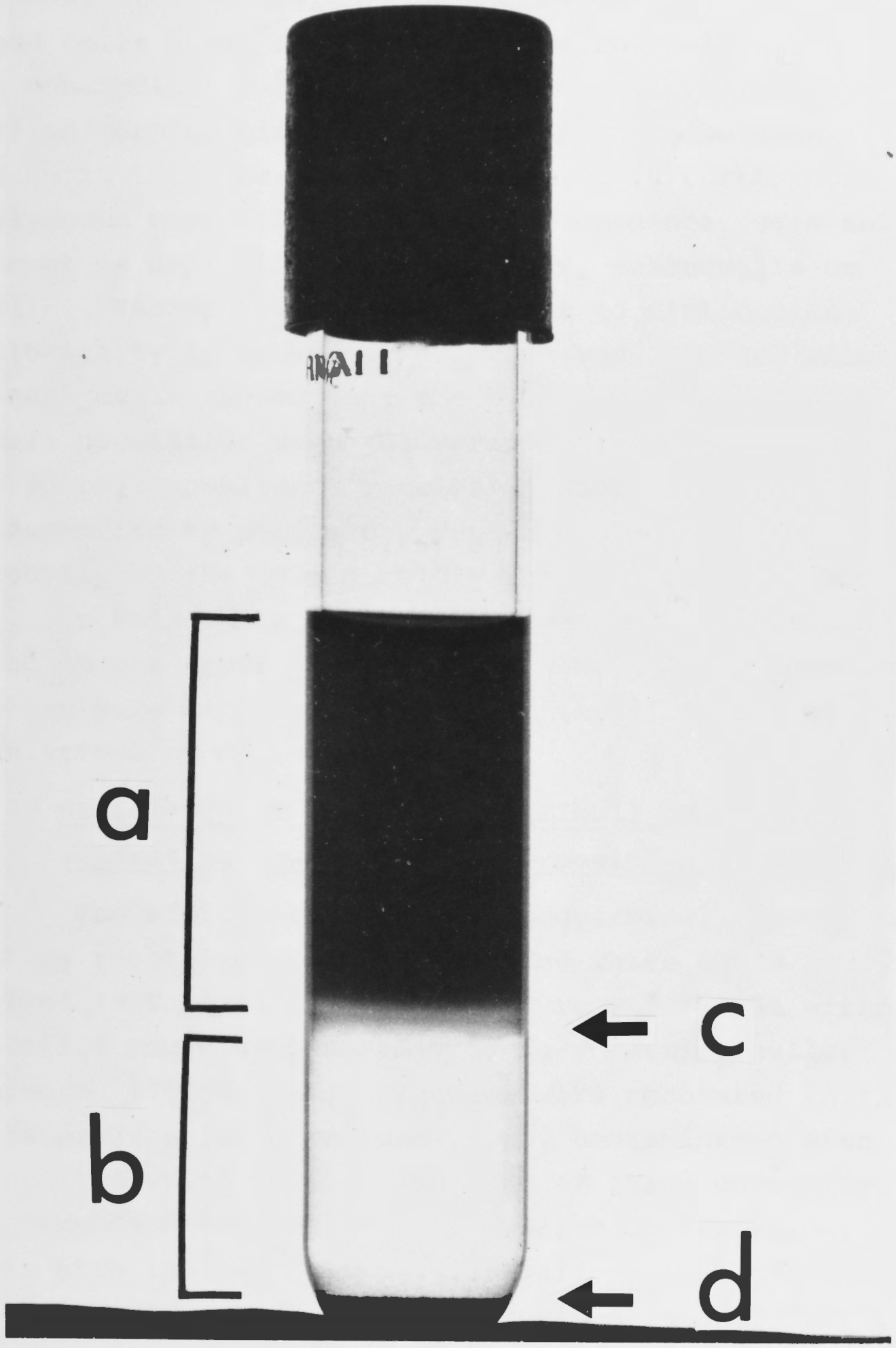
c) As assessed by morphology. Stem cells were also assayed by the spleen colony assay of Till and McCulloch, (1961) and were shown to be completely recovered in the upper layer (Figure 5.2).

Figure 5.1

Separation of rosetting and non-rosetting cells on Isopaque/ Ficoll.

a) F15 culture medium.
b) Isopaque/Ficoll mix.
c) The non-rosetted white cell layer at the interface ("upper layer").
d) SRBC and rosetted lymphocytes in the pellet ("lower layer").

This was the appearance immediately after centrifugation (2000g, 20min at 20°C). For photographic purposes, separation was carried out in a glass centrifuge tube.


a
b
c
d

cells (9%) sedimented but almost half of these cells were dead as assessed by trypan blue exclusion; in fact most of the dead cells applied appeared in the red cell pellet. Stem cells, mononuclear cells and polymorphonuclear leucocytes were identified morphologically and were shown to be quantitatively recovered in the upper layer (Figure 5.2). Furthermore, with the polymorph population there was no evidence for a selective enrichment or depletion of neutrophils, eosinophils or basophils. Macrophages were difficult to distinguish morphologically in mouse spleen. However, studies with peritoneal cells showed that the bulk of the macrophages in this cell population were recovered in the upper layer (Parish and Davidson, unpublished results). Stem cells were not only identified by morphology but were also assayed functionally by the spleen colony assay of Till and McCulloch, (1961). In both cases it was found that all the stem cells appeared in the upper layer (Figure 5.2). It is noteworthy that there were excellent recoveries (>95%) of all of the subpopulations of cells identified.

5.3.1.2 Efficiency of the Isopaque/Ficoll system in separating Ig-bearing and θ-positive lymphocytes

The efficiency of the Isopaque/Ficoll system in separating rosetting and non-rosetting white cells is summarized in Table 5.2. The procedure was >99.5% efficient at depleting mouse spleen cells of Ig-rosetting cells. Furthermore, 97% of the Ig rosettes were recovered in the lower layer in a 76% pure form, i.e., contaminated with 24% of unrosetted white cells. The bulk of these unrosetted cells (>70%) were dead as assessed by trypan blue exclusion. In contrast with the Ig-rosettes, virtually all of the θ-positive lymphocytes (91%) were recovered in the upper layer. It is generally accepted that mouse "B" lymphocytes have a high density of surface Ig (Raff et al., 1970a; Pernis et al., 1970; Rabellino et al., 1971) whereas mouse "T" lymphocytes carry the theta isoantigen (Raff, 1970a, b). Thus this system represents an excellent method for separating mouse T and B cells. Subsequent functional studies reported in this chapter confirmed this separation. For simplicity, the non-Ig bearing (upper layer) cells are henceforward termed T cells and the

Figure 5.2

Upper:

A smear (Giemsa stained) of the upper layer of an Ig rosette depleted spleen cell suspension. (Scale in microns). Mononuclear cells and polymorphonuclear leucocytes could be identified morphologically and were shown to be recovered quantitatively in the upper layer. 60-70% of these cells were sensitive to treatment with anti-θ ascitic fluid and complement.

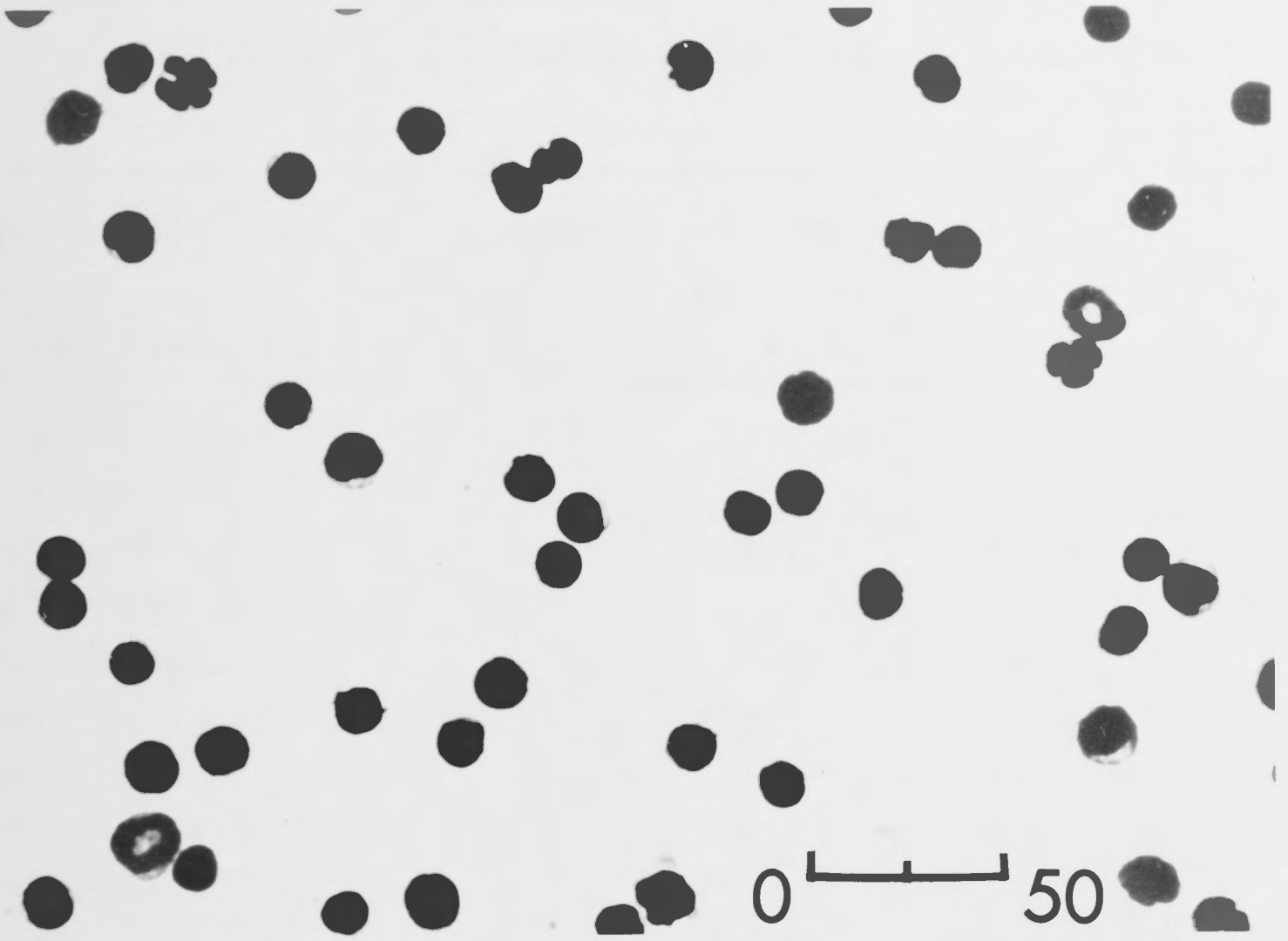
Lower:

The colony forming unit (CFU) assay of Till and McCulloch. The assay method was based on the fact that the intravenous injection of stem cells into syngeneic, lethally irradiated (850 rads) hosts led to the formation of colonies of proliferating cells in the spleens of these animals. The colonies appeared as gross nodules in the spleen which could be readily counted. They were discrete, round or oval, and greyish in colour, embedded in the red mass of the spleen. CFU were counted 10 days after cell transfer. Mice received either no cells, or $1x10^7$, $1x10^6$ or $1x10^5$ unfractionated spleen cells or upper layer cells respectively.

a) The appearance of a spleen from a mouse which received 1×10^7 upper layer cells.

b), c) and d) The appearance of spleens from mice which received 1×10^7, 1×10^6 and 1×10^5 unfractionated spleen cells respectively.

e) The appearance of a spleen from a mouse into which no cells were transferred. Note the appearance of a CFU (arrowed). There was generally one per spleen of host origin 10 days after irradiation.


0
50


a
b
c
d
e
1
2

Table 5.2: Efficiency of Isopaque/Ficoll system in separating Ig-Bearing and θ-positive lymphocytes

Original spleen cell population:	θ^+ lymphocytes = 19.0% Ig rosettes = 44.8%	
Fractionations (%)	E b)	E anti-Ig b)
Upper layer c)	77.2(98)	26.0(99) d)
Lower layer c)	10.5(52)	60.0(74)
Recovery	87.7	86.0
% lymphocytes as Ig rosettes in lower layer	-	76
Recovery in Ig rosettes in lower layer	-	97
Ig rerosetting of upper layers	46	<0.1
% lymphocytes θ^+ in upper layers	24	66
Recovery of θ^+ lymphocytes in upper layers	98	91

a) In each fractionation 1 x 10^8 mouse spleen cells were applied to the Isopaque/Ficoll.

b) E = erythrocytes; E anti-Ig = erythrocytes coated with anti-Ig for Ig rosetting.

c) Upper layer = non-rosetting lymphocytes; lower layer = red cells + rosetting lymphocytes.

d) Values in parentheses represent % viability of cell populations as assessed by trypan blue exclusion.

Ig-bearing (lower layer) cells are termed B cells.

5.3.1.3 Radioautography on upper layer cells

To further substantiate that depletion of Ig-rosettes removed all lymphocytes with a high density of surface Ig, the undepleted and depleted spleen cell populations were examined for surface Ig by radioautography (Figure 5.3). First, it was found that running spleen cells on Isopaque/Ficoll with unsensitized red cells resulted in little or no change in both the cell surface density of Ig and the incidence of Ig-bearing cells detected by radioautography (compare Fig. 5.3a with 5.3c). However, when spleen cells were incubated with the concentration of rabbit anti-mouse Ig used for rosetting some surface Ig was blocked and so a reduction in both the surface density of Ig and the frequency of Ig-bearing cells detected by radioautography was observed (compare Fig. 5.3a with 5.3d). These reductions were much less marked if radioautographs were exposed for longer periods i.e., 96h rather than 24h. Depletion of Ig-rosetting cells resulted in a dramatic reduction in the incidence of Ig-bearing cells detected by radioautography (>97%, compare Fig. 5.3b and 5.3d and inset a and b). Furthermore, the great majority of Ig-bearing cells which escaped depletion possessed a very low density of surface Ig. It was estimated that such lymphocytes carried <500 Ig molecules on their surface (Ada et al., 1966).

An experiment was carried out to determine whether the low density Ig-bearing cells which escaped depletion were either T cells or B cells. Spleen cells depleted of Ig-rosettes were labelled with ^{125}I anti-light chain globulin and then treated with anti-θ ascitic fluid and complement. Cells lysed by the antiserum were detected on radioautographs by the nigrosin staining procedure (2.2.11). It was found that from 68-83% of the labelled cells were killed by anti-θ antiserum and complement. Thus it appeared that the bulk of the low density Ig-bearing cells which escaped depletion were T lymphocytes.

Figure 5.3

Estimation by radioautography of the frequency of Ig-bearing cells in spleen cell populations before and after removal of Ig rosettes on Isopaque/Ficoll.

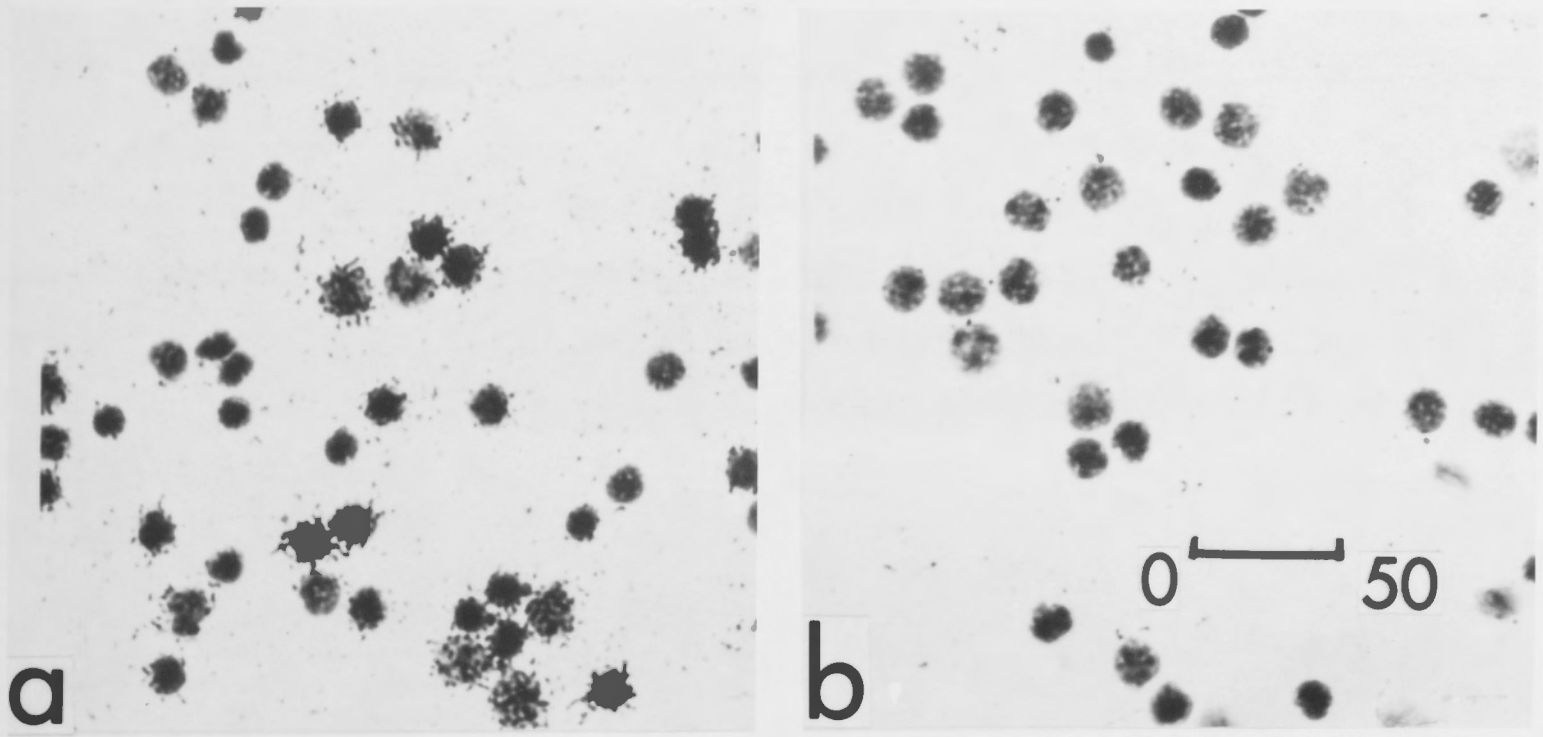
a) Untreated spleen cells - 58.8% Ig-bearing cells after 96h exposure; 54.3% Ig-bearing cells after 24h exposure.

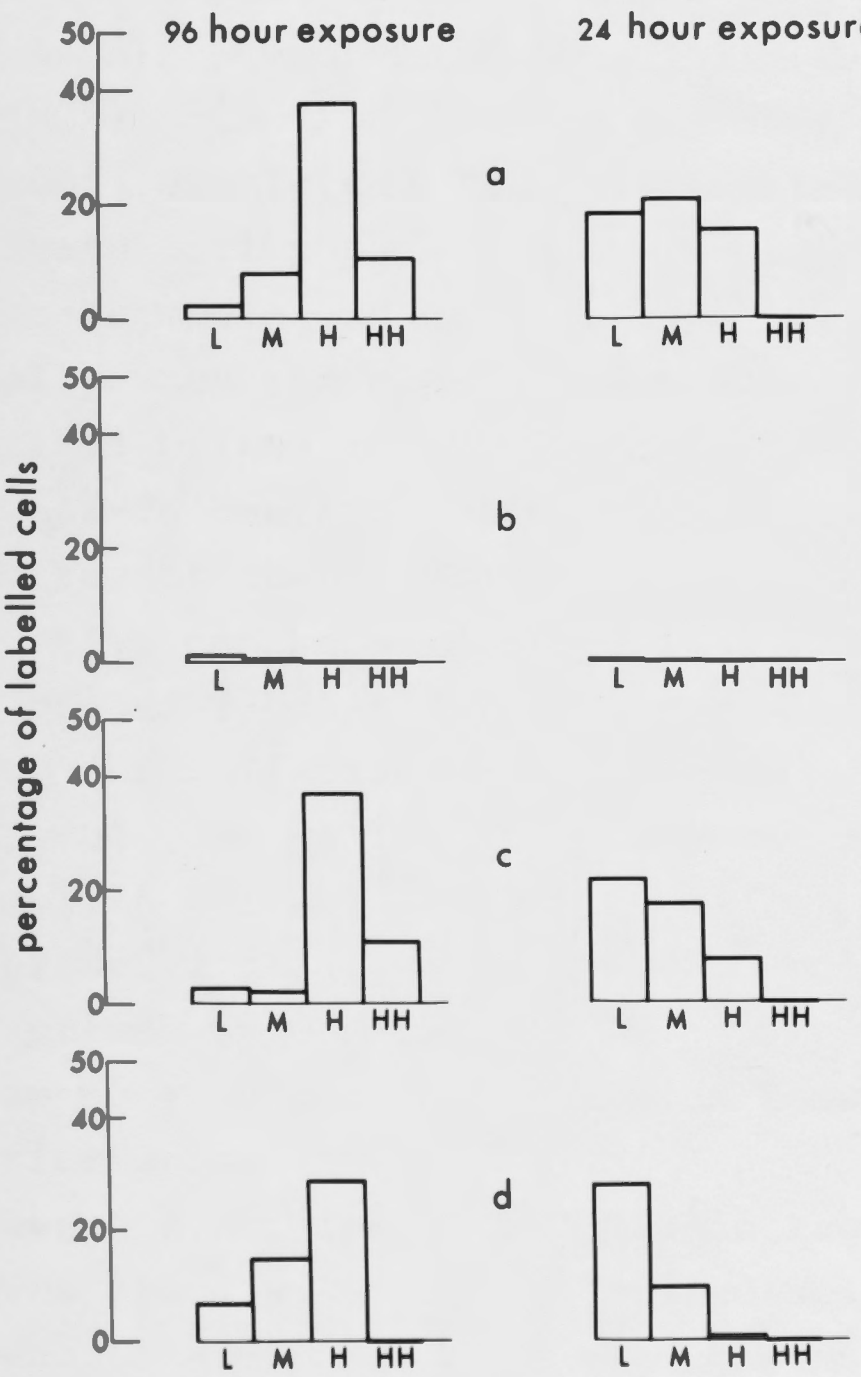
b) Spleen cells depleted of Ig-rosettes by Isopaque/Ficoll procedure - 1.8%; 0.4%.

c) Spleen cells run on Isopaque/Ficoll with non-Ig-rosetting erythrocytes - 52.9%; 46.6%.

d) Spleen cells pretreated with rabbit anti-mouse Ig - 50.5%; 38.0%.

The upper inset shows the appearance of radioautographs a) and b) after 96h exposure. (Giemsa stained; scale in microns). A dramatic reduction in the incidence of Ig bearing cells is apparent.


a
b
0
50


96 hour exposure
24 hour exposure
percentage of labelled cells
50
40
20
0
L M H HH
a
b
c
d

5.3.2 Immunological responsiveness of separated spleen cell populations

Two antibody responses were used to assess the immunological responsiveness of the separated spleen cell populations. The first system measured the anti-hapten response to DNP-HCY *in vitro* and the second measured the anti-hapten response to DNP-MON *in vitro* and *in vivo*. (See Chapter 3).

5.3.2.1 Response to DNP-HCY *in vitro*

The capacity of unfractionated and fractionated spleen cells to respond to DNP-HCY *in vitro* is summarized in Table 5.3. As has been demonstrated (3.3.2.1.D) a mixture of carrier (HCY) primed and hapten (DNP-BSA) primed spleen cells produced a significantly higher anti-DNP response than the two separate cell populations. In contrast, spleen cells primed to the heterologous carrier (HGG) did not act synergistically with the DNP-BSA primed spleen cells. The cell populations primed to DNP-BSA and HCY were then separated into Ig and non-Ig bearing cells on Isopaque/Ficoll and various mixtures of these subpopulations examined for synergism. The results can be summarized as follows:

a) Carrier primed T cells (i.e., non-Ig-bearing cells) were much more effective than carrier primed B cells (i.e., Ig-bearing cells) at collaborating with hapten primed spleen cells.
b) Hapten primed T cells were unable to collaborate with carrier primed spleen cells.
c) There was no evidence for synergism between hapten and carrier primed B cells.
d) The greatest synergism occurred when hapten primed B cells were mixed with carrier primed spleen cells.

These results are consistent with the notion that T cells primed to the carrier, HCY, collaborate with B cells primed to the hapten, DNP, in the *in vitro* anti-DNP antibody response to DNP-HCY.

5.3.2.2 Response to DNP-MON *in vitro*

It has already been shown (3.3.2.3) that the anti-

Table 5.3: Collaboration between Isopaque/Ficoll purified T cells and B cells in the in vitro anti-DNP antibody response to DNP-HCY

Cells cultured [a]	Number per culture	Anti-DNP PFC per culture [b]
DNP-BSA primed spleen	2×10^6	234 ± 14
HCY primed spleen	5×10^6	94 ± 12
DNP-BSA primed spleen + HCY primed spleen	2×10^6 + 5×10^6	604 ± 67
DNP-BSA primed spleen + HGG primed spleen	2×10^6 + 5×10^6	232 ± 4
DNP-BSA primed spleen + HCY primed T cells [c]	2×10^6 + 2.5×10^6	533 ± 74
DNP-BSA primed spleen + HCY primed B cells [c]	2×10^6 + 5×10^6	315 ± 44
DNP-BSA primed T cells + HCY primed spleen	2×10^6 + 5×10^6	168 ± 4
DNP-BSA primed B cells	2×10^6	184 ± 20
DNP-BSA primed B cells + HCY primed spleen cells	2×10^6 + 5×10^6	1143 ± 64
HCY primed B cells	2×10^6	<10
HCY primed B cells + DNP-BSA primed B cells	2×10^6 + 2×10^6	186 ± 10

a) Cultures were incubated with 2 µg/ml DNP-HCY.

b) PFC assays were made 4 days after culture. Each value is the arithmetic mean of four cultures ± the standard error of the mean.

c) B cells = lower layer from Isopaque/Ficoll separation of Ig-bearing and θ-positive lymphocytes.
T cells = upper layer from separation.

DNP response to DNP-MON was highly T cell dependent, anti-θ treated spleen cells giving a 3-7 fold lower antibody response *in vitro* than untreated spleen cells or spleen cells treated with normal AKR serum and complement (also see Table 5.4). It was found that spleen cells depleted of Ig-bearing cells by the Isopaque/Ficoll system were unable to produce an *in vitro* anti-DNP antibody response to DNP-MON. However, these cells were capable of completely reconstituting the anti-DNP response of anti-θ treated spleen cells (Table 5.4), a result consistent with the T cell content of this Isopaque/Ficoll preparation.

A second series of experiments measured the response of both the upper and lower layer cells from the Isopaque/Ficoll to DNP-MON (Table 5.5). As in the previous experiment the upper layer cells (i.e., T cells) produced little or no antibody to DNP. However the lower layer preparation (i.e., B cells) gave a substantial antibody response, although this response was significantly lower than that given by the untreated spleen cells. Treatment of the lower layer with anti-θ antiserum and complement significantly reduced the anti-DNP response. As a control to test for non-specific leakage of some functional T cells through the Isopaque/Ficoll mix, the lower layers from control rosetted spleen cells were added to anti-θ treated spleen cells. No restoration of the anti-DNP response was obtained. Hence there were no functional T cells in these lower layers (Table 5.6). From these results it was concluded that there were some T cells which carried enough surface Ig to form Ig rosettes and which were contaminating the lower layer of Ig positive cells. Recombination of the upper and lower layers resulted in a response equal to or greater than the unfractionated spleen cell population.

5.3.2.3 Response to DNP-MON *in vivo*

A similar experiment to the *in vitro* experiment reported in Table 5.5 was carried out *in vivo*. Unfractionated and fractionated cells were transferred intravenously into irradiated recipients (850 rads 24 h previously) with 5 μg DNP-MON. Six days later spleens were harvested and the

Table 5.4: Restoration of *in vitro* anti-DNP antibody response of anti-θ treated spleen cells by Isopaque/Ficoll purified T cells

Cells cultured a)	Anti-DNP PFC per culture b)			
	Expt.1	Expt. 2	Expt. 3	Expt. 4
Untreated spleen	565±59	1193±117	1303±87	1275±78
Spleen cells treated with normal AKR ascitic fluid and C'	635±68	1185±41	n.d.[c]	n.d.
Spleen cells treated with anti-θ ascitic fluid and C'	105±21	365±39	390±48	217±35
T cells [d]	<20	0	<50	<50
Anti-θ treated cells + T cells	677±41	1193±81	1120±68	1625±129

a) 5×10^6 *viable* cells of each type were cultured per well, except in experiment 1, where 4×10^6 cells were cultured. DNP-MON was at a final concentration of 100 ng/ml.

b) Cultures were assayed on day 3. Each value is the arithmetic mean of 4 cultures ± the standard error of the mean.

c) Not done.

d) Upper layer from Isopaque/Ficoll separation of Ig-bearing and θ-positive lymphocytes (Table 5.2).

Table 5.5: Collaboration between Isopaque/Ficoll purified T cells and B cells in the in vitro anti-DNP antibody response to DNP-MON

Cells cultured [a]	Anti-DNP PFC per culture [b]	
	Expt. 1	Expt. 2
Untreated spleen	1193±117	1665±115
B cells	828±42	885±39
Anti-θ treated B cells	n.d.[c]	325±22
T cells	0	20±15
B cells + T cells [d]	1048±38	2134±159

a) 5×10^6 viable cells of each type were cultured per well. DNP-MON was at a final concentration of 100 ng/ml.

b) Cultures were assayed on day 3. Each value is the arithmetic mean of 4 cultures ± the standard error of the mean.

c) Not done.

d) B cells = lower layer from Isopaque/Ficoll separation of Ig-bearing and θ-positive lymphocytes (Table 5.2).
T cells = upper layer from same separation.

Table 5.6: Control to test for non-specific sedimentation of functional T cells.

Cells cultured [a]	Anti-DNP PFC per culture [d]
Untreated spleen	1180±8
Anti-θ treated spleen	440±18
Anti-θ treated spleen + lower layer control [b] rosetted cells	415±22
Anti-θ treated spleen + T cells [c]	1185±76
Lower layer control rosetted cells alone	45±10

a) 5×10^6 viable untreated or anti-θ treated cells and approximately 2×10^6 lower layer control rosetted cells were cultured per well as described.

b) Spleen cells + SRBC were spun on Isopaque/Ficoll, the SRBC lysed by hypotonic shock and the recovered cells cultured.

c) T cells = upper layer from Isopaque/Ficoll separation of Ig-bearing and θ-positive lymphocytes; 4×10^6 per well.

d) Each value represents the arithmetic mean of 4 cultures ± the standard error of the mean.

anti-DNP PFC response measured (Table 5.7). Again the upper layer preparation produced little or no antibody to DNP. The lower layer produced some, but this was significantly lower than that produced by the unfractionated spleen cells. Recombination of the upper and lower layers resulted in a response equal to that of the unfractionated spleen (see also Tables 7.6 and 7.7).

5.3.3 Fc rosetting lymphocytes

5.3.3.1 Fractionation of Fc and non-Fc receptor lymphocytes on Isopaque/Ficoll

The rosetting procedure was also adapted for depleting and obtaining in pure form Fc receptor lymphocytes (FcRL) from mouse spleen cells. 40-50% of spleen cells formed Fc rosettes. The separated cell populations were examined for their ability to respond to DNP-MON *in vitro* (Table 5.8). The non-FcRL gave only a slight response above background but the Fc positive (lower layer) gave a significant response, 2-3½ fold above background. This response was removed by treating the cells with anti-θ and complement prior to culture. These functional helper cells were specifically forming rosettes, as shown by the data in Table 5.6. Hence, it was concluded that there was a subpopulation of theta positive, helper cells which possessed a receptor for the Fc portion of Ig. Recombination of FcRL and non-FcRL populations led to anti-DNP responses of the same magnitude as unfractionated spleen.

5.3.3.2 Fc receptor helper cells

The presence of Fc bearing, functional helper cells was further demonstrated by a double rosetting experiment where spleen cells were first depleted of Ig RFC and the upper, T layer was then re-rosetted for FcRL. Approximately 7% of the T layer formed Fc rosettes. On a cell per cell basis the Fc depleted T layer was less effective than the undepleted T layer at restoring the anti-DNP response to anti-θ treated spleen cells. The $Fc^{+}Ig^{-}$ cells were as good as $Fc^{+}Ig^{-}$ cells as helper cells in the anti-DNP response (Table 5.9). Thus these results suggest that a substantial proportion of functional helper cells possess an Fc receptor.

Table 5.7: Collaboration between Isopaque/Ficoll purified T and B cells in the *in vivo* anti-DNP response to DNP-MON

Cells transferred [a]	Anti-DNP PFC per spleen [b]	
	Direct	Indirect
None	14±2	4±4
Untreated spleen	2308±216	3648±640
B cells	300±53	388±115
T cells	44±9	84±49
B cells + T cells [d]	2408±384	4332±1330

a) 10^7 viable lymphocytes were injected into each mouse.

b) Anti-DNP PFC were measured on day 6 after cell transfer. Each value represents the arithmetic mean of five spleens ± the standard error of the mean.

c) Indirect plaques were developed using a rabbit anti-mouse Ig serum at an appropriate dilution (1/20) (Cunningham and Szenberg, 1968).

d) B cells = lower layer from Isopaque/Ficoll separation of Ig-bearing and θ-positive lymphocytes.
T cells = upper layer from same separation.

Table 5.8: The response of spleen cells fractionated by Fc rosetting and separation on Isopaque/Ficoll to DNP-MON in vitro

Cells cultured [a]	Anti-DNP PFC per culture [c] Expt. 1 [b]	Expt. 2 [b]
Unfractionated spleen	1375±82	2390±176
Fc^- cells alone (Upper layer)	143±45	365±53
Fc^+ cells alone (Lower layer)	650±37	585±142
Fc^- + Fc^+ cells	1450±20	1995±204
Anti-θ Fc^+ cells	283±20	195±40

a) 5×10^6 unfractionated cells and 2.5×10^6 viable Fc^-, Fc^+ and anti-θ treated Fc^+ were cultured as described.

b) In experiment 1, 44.1% of spleen cells were Fc^+.
In experiment 2, 48.9% of spleen cells were Fc^+.

c) Each value represents the arithmetic mean of 4 cultures ± the standard error of the mean.

Table 5.9: Demonstration of functional helper cells with Fc receptors in upper layer of Ig depleted spleen (T cell layer)

Cells cultured	Number per culture	Anti-DNP PFC per culture [c]
Untreated spleen	5×10^6	1290 ± 48
Anti-θ treated spleen	5×10^6	355 ± 48
Anti-θ treated spleen + T cells [a]	5×10^6 + 0.5×10^6	650 ± 45
Anti-θ treated spleen + T cells	5×10^6 + 1.5×10^6	815 ± 87
Anti-θ treated spleen + T cells	5×10^6 + 3×10^6	1000 ± 55
Anti-θ treated spleen + T cells	5×10^6 + 5×10^6	1185 ± 76
Anti-θ treated spleen + Fc^- T cells [b]	5×10^6 + 0.5×10^6	455 ± 82
Anti-θ treated spleen + Fc^- T cells	5×10^6 + 1.5×10^6	660 ± 93
Anti-θ treated spleen + Fc^- T cells	5×10^6 + 3×10^6	665 ± 39
Anti-θ treated spleen + Fc^+ T cells	5×10^6 + ~2×10^6	690 ± 41

a) T cells = upper layer from Isopaque/Ficoll separation of Ig-bearing and θ-positive lymphocytes.

b) T cells were re-rosetted and depleted of Fc receptor lymphocytes. In this experiment 6.5% of the Ig^- cells formed Fc rosettes.

c) Each value is the arithmetic mean of 4 cultures ± the standard error of the mean.

5.4 DISCUSSION

In this chapter a one step procedure for fractionating and obtaining in pure form Ig-bearing cells and an enriched population of theta-positive cells from mouse lymphoid populations was described. Virtually all theta-positive lymphocytes (> 90%) were recovered in the non-rosetting population; few sedimented with the Ig rosettes (Table 5.2). Helper T cells appeared in the non-rosetting population whereas the precursors of antibody forming cells (i.e. B cells) were isolated in the preparations of Ig rosettes. This was observed in the *in vitro* anti-hapten response to DNP-HCY and DNP-MON (Tables 5.3, 5.4 and 5.5) and the *in vivo* response to DNP-MON (Table 5.7). It was demonstrated that a pure population of carrier primed T cells could collaborate with a pure population of hapten primed B cells to generate an anti-hapten response. There is ample indirect evidence for this collaboration (Wigzell et al., 1972; Raff, 1970b; Mond et al., 1972; Niederhuber and Möller, 1973), but the above results represented a direct demonstration of this phenomenon and confirmed conclusively the T dependent nature of the anti-DNP response to DNP-MON described in Chapters 3 and 4.

In these studies some evidence was obtained which suggested that a small subpopulation of Ig-bearing T cells existed. Firstly, a small number (<1%) of T cells were found which possessed a low density of surface Ig (Figure 5.3). Secondly, the Ig-rosettes produced a small antibody response to DNP-MON which was sensitive to anti-θ treatment. Other workers have detected peripheral T cells with surface Ig (e.g. Hämmerling and Rajewsky, 1971; Bankhurst et al., 1971; Goldschneider and Cogen, 1973; and see 1.1.1). However, whether these cells actively synthesize their surface Ig (Roelants et al., 1974) or passively acquire it via an Fc receptor (Modabber and Coons, 1972; Yoshida and Andersson, 1972; Orr and Paraskevas, 1973; Webb and Cooper, 1973; Tables 5.8 and 5.9) remains to be established. Certainly the results suggested that such cells can act as helpers but it is not known whether all helper cells are contained within this subpopulation.

The Isopaque/Ficoll separation procedure is a highly

versatile technique as any subpopulation of lymphocytes which can be identified by rosetting can be purified using this method. The procedure was used to deplete and purify Fc receptor lymphocytes. It was shown that there is a population of theta-positive, helper cells with Fc receptors. Also, functional helper cells with Fc receptors could be demonstrated in the Ig depleted T layer. $Fc^{+}Ig^{-}$ lymphocytes have also been found in rat thoracic duct lymph (Parish and Hayward, 1974b) but owing to the lack of a reliable T cell marker in the rat it had not been demonstrated whether these were T or B lymphocytes. The results with the mouse lymphocytes suggest that some, or all, of these cells are T cells.

5.5 SUMMARY

A procedure for both depleting and obtaining in pure form Ig-bearing cells from mouse lymphoid populations was described. The Ig-bearing cells were identified by rosetting and the rosettes separated from the non-rosetting lymphocytes by centrifugation on Isopaque/Ficoll. The rosettes sank with the red cells and the non-rosetting lymphocytes floated. The procedure was >99.5% efficient at depleting mouse lymphoid population of Ig-rosetting cells and was also >97% efficient at removing the Ig-bearing cells detected by radioautography. Furthermore, after lysis of the red cells by hypotonic shock the Ig-bearing cells were recovered in a highly pure form. The total recovery of white cells and rosettes applied was >85%.

This procedure was shown to produce a functional separation of T and B lymphocytes. The cell population depleted of Ig-rosettes behaved as a pure T cell preparation. It lacked precursors of antibody forming cells, but contained virtually all of the θ-positive lymphocytes, the bulk of the helper cells detected in two anti-hapten antibody responses. In contrast, the preparation of Ig-rosettes expressed B cell properties. This population contained all of the antibody forming cell precursors. However, there was some evidence that there was a small subpopulation of T cells which possessed surface Ig and were present in the Ig positive

rosette population.

A modification of the separation procedure enabled fractionation of Fc receptor bearing and non-Fc receptor bearing lymphocytes. It was shown that there was also a small subpopulation of T cells which possessed an Fc receptor.

The Ig rosetting and separation was used to demonstrate formally that carrier primed T cells collaborate with hapten primed B cells both in vivo and in vitro to generate an anti-hapten antibody response to a hapten-carrier conjugate, using the responses to DNP-HCY and DNP-MON.

CHAPTER 6

The requirement of B cells for release of a specific T cell replacing factor

6.1 INTRODUCTION

The mechanisms by which T and B cells collaborate in antibody production are still a matter of controversy. As discussed in 1.2, the various models proposed can be divided broadly into the contact models, where cell-cell contact is a necessary requirement for B cell stimulation and the factor models. Variants of this latter model postulate that T cells, once activated by antigen, release either short range non-specific factors which trigger B cells already stimulated by antigen, or that T cells activated by antigen release specific factors (antigen-receptor complex?) which can then activate B cells directly, or that both specific and non-specific factors are produced. However, where specific factors have been characterized, agreement has not been reached as to their chemical nature (Feldmann, 1972c; Taussig and Munro, 1974). In addition, in all cases where specific factors have been found, the ATC have been primed *in vivo* and hence the contribution of antibody, either passively acquired by the ATC (Hunt and Williams, 1974; Hudson et al., 1974) or synthesized by contaminating B cells from the donor thymus population or the residual radio-resistent host B cells (Parish and Hayward, 1974c; Pilarski and Cunningham, 1974) needs to be rigorously eliminated. The frequent observation that antibody against one antigenic determinant enhances the response to another unrelated determinant on the same antigen (e.g. Schierman et al., 1969; Pincus et al., 1973; Janeway and Paul, 1973) further reinforces the possibility that the carrier-specific factors released by ATC may be B cell derived immunoglobulin.

The advantage of being able to obtain pure populations of T and B lymphocytes (Chapter 5) and the ready adaptation of the T dependent *in vitro* anti-hapten response to DNP-MON (Chapter 3) to culture in a double chamber system enabled a study of the ability of T cells to collaborate with B cells across a cell impermeable Millipore membrane.

It will be shown in this chapter that T cells alone, under the experimental conditions used, were not able to collaborate with B cells across a Millipore membrane. However, B and T cells together produced a specific collaborative factor which passed through the membrane and

partially restored the B cell response. In Chapter 7 the importance of the carrier specific (i.e. flagellin specific) B cells in the production of this collaborative factor will be demonstrated.

6.2 MATERIALS AND METHODS

6.2.1 Animals

CBA/H mice, 7-9 weeks of age, obtained from breeding colonies in this School were used throughout.

6.2.2 Antigens

DNP-flagellin ($DNP_{1.5}MON$) was prepared as described earlier (3.2.3). Sheep red blood cells (SRBC) were obtained from Commonwealth Serum Laboratories, Melbourne, and were washed four times in normal saline before use. SRBC primed mice were given $2x10^8$ SRBC, i.v., 7 weeks prior to use.

6.2.3 Fractionation of spleen cells on Isopaque/Ficoll

Spleen cell suspensions were fractionated into T and B populations by the one-step procedure described in Chapter 5.

6.2.4 Tissue culture

Culture medium was as described earlier (3.2.5). Cells were cultured in this medium with antigen (usually DNP-MON at 100 ng/ml) in 16mm Linbro trays (Linbro Chemical Co., New Haven, Connecticut, U.S.A.) or in a modified Marbrook - Diener culture system consisting of an inner chamber and an outer chamber (Figure 6.1). The bottom end of the inner chamber was sealed off from the outer chamber by a Millipore membrane (Millipore Corporation, Bedford, Massachusetts, U.S.A. - 0.45μ pore size (6.1b) or this type of membrane was replaced by a dialysis membrane (Union Carbide Corp., New York) fixed on by siliconized rubber rings (6.1a). Cells were cultured in these double chambers with 5×10^6 cells in 1.0 ml in the upper chamber and 5×10^6 cells in 2.0 ml in the lower chamber. Cultures were placed in a humidified incubator at 37°C in an atmosphere of 10% CO_2, 7% O_2 in N_2 for three days and then harvested and assayed for

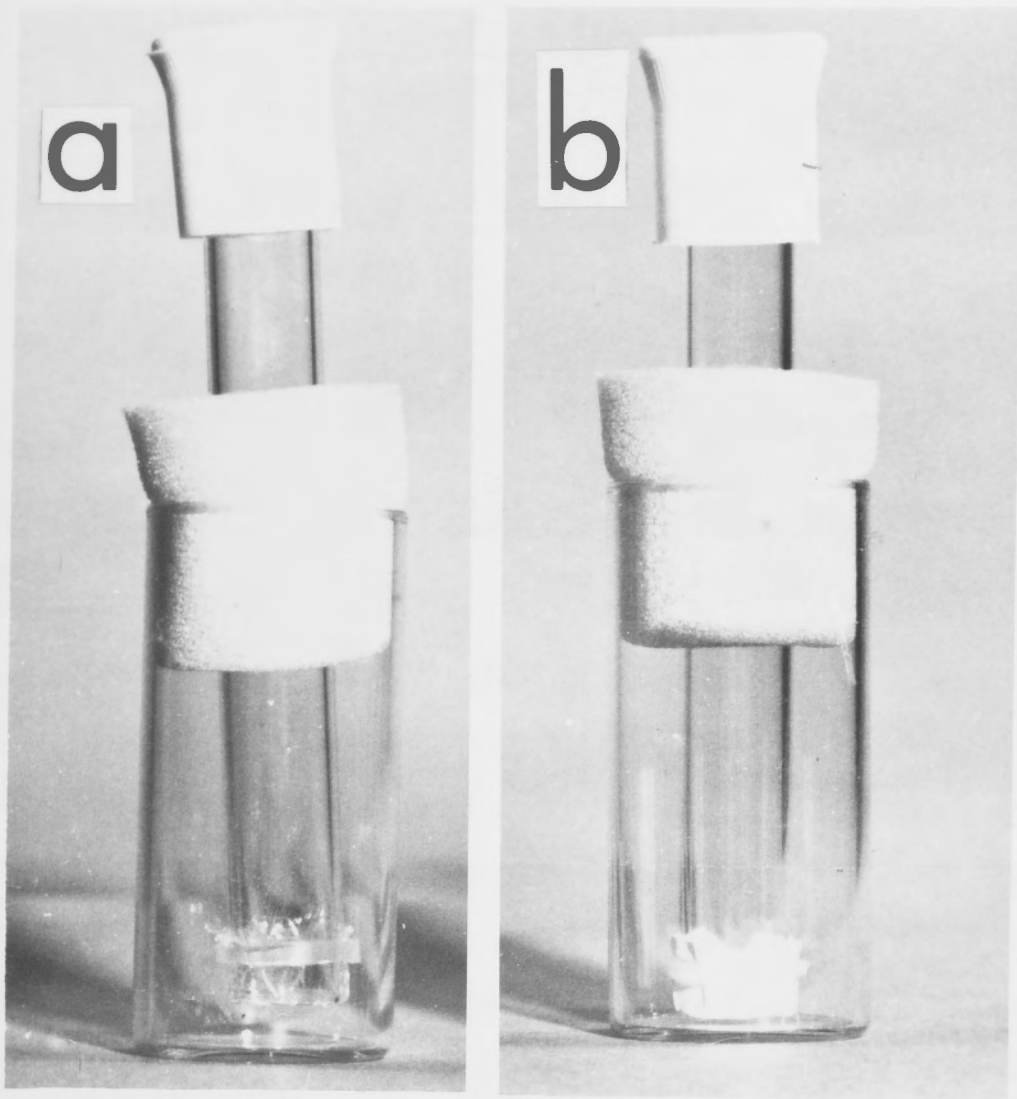


Figure 6.1

Double chamber culture vessels for examining the ability of cells to collaborate across cell impermeable membranes.

a) The bottom end of the inner chamber was sealed off from the outer chamber by a dialysis membrane.

b) The bottom end of the inner chamber was sealed off from the outer chamber by a Millipore membrane (0.45µ pore size).

In both cases membranes were fixed on by siliconized rubber rings.

Cells were cultured in these double chambers with 5×10^6 cells in 1.0 ml in the upper chamber and 5×10^6 cells in 2.0 ml in the lower chamber.

plaque-forming cells.

6.2.5 Enumeration of plaque-forming cells

See 3.2.14

6.2.6 Treatment of cells with anti-θ and complement

See 3.2.16

6.3 RESULTS

6.3.1 Summary of the characteristics of the response to DNP-MON in vitro

It has already been shown (Chapters 3, 4 and 5) that the anti-hapten response to DNP-MON is highly T cell dependent. Mice used in these experiments were naturally pre-primed to MON and thus spleen cells gave an excellent response to this antigen in vitro. Table 6.1 summarizes the T dependent nature of the response discussed in previous chapters. Anti-θ treated spleen cells gave a 3-7 fold lower plaque-forming cell response compared to untreated spleen cells or spleen cells treated with normal ascitic fluid and complement when equal numbers of viable cells were cultured. The response could be fully restored by addition of the T cell fraction of spleen following removal of the Ig positive rosette-forming cells on Isopaque/Ficoll.

The Ig positive cells, when cultured alone, gave a substantial antibody response, although this response was significantly lower than that given by the untreated spleen cells. Treatment of these cells with anti-θ ascitic fluid and complement significantly reduced the anti-DNP response. It was thus concluded that the Ig positive population was contaminated with some functional T cells with enough surface Ig to form rosettes. Recombination of the T and B layers resulted in a response equal to, or greater than, the unfractionated spleen cell population.

It was also demonstrated that DNP-MON behaved as a classic hapten-carrier, chemical linkage between the hapten and carrier being required for the anti-hapten response to be induced (Table 3.15). There was no evidence that carrier activated T cells released a mitogen which non-specifically

Table 6.1 : T-cell dependence of the anti-DNP response to DNP-MON.

Cells Cultured [a]			Direct anti-DNP PFC per culture [c]
Unfractionated spleen	Bottom Layer (B) Cells [b]	Top Layer (T) Cells [b]	
Untreated	-	-	1430±115
Normal ascitic fluid and C'	-	-	1185±41
Anti-θ ascitic fluid and C'	-	-	365±39
Anti-θ ascitic fluid and C'	-	+	1372±98
-	-	+	<5
-	+	-	828±42
-	+	+	1048±38
-	anti-θ treated [d]	-	325±22

a) 5×10^6 viable cells were cultured per well.

b) Isopaque/Ficoll fractionated cells.

Bottom layer: Ig positive RFC.
Top layer: The T enriched layer.

c) Each value is the arithmetic mean of four cultures ± the standard error of the mean. DNP-MON was at a final concentration of 100 ng/ml.

d) Bottom layer cells were treated with anti-θ and C' and 5×10^6 viable cells cultured per well.

triggered hapten-specific B cells.

6.3.2 Transfer of a T cell replacing factor across a Millipore membrane

The *in vitro* response to DNP-MON was used in conjunction with the Isopaque/Ficoll separation procedure to look at cellular collaboration across a Millipore membrane.

6.3.2.1 Partial restoration of the anti-DNP response of anti-θ treated spleen across a Millipore membrane

Initially normal spleen cells (i.e. B and T cells) were cultured in the upper chamber and the response in the lower chamber of anti-θ treated spleen cells (B cells) to DNP-MON measured. DNP-MON was present in both chambers (Table 6.2). Partial restoration of the anti-DNP response was obtained in all experiments, the average restoration being around 40% and varying in individual experiments from 30-90%.

6.3.2.2 The effect of culturing fractionated cells in the upper chamber on production of the factor

The next step was to fractionate spleen cells by the Isopaque/Ficoll procedure and culture either pure T cells in the upper chamber or anti-θ treated spleen (B cells) in the upper chamber. It was found (Table 6.3) that both B and T cells were required in the upper chamber for the collaborative factor to be produced. Purified T cells or B cells cultured alone in the upper chamber had no, or only a very slight (<2% restoration), effect on the anti-DNP response of the anti-θ treated spleen cells cultured in the lower chamber. However, if the T cells were cultured in the lower chamber with the anti-θ treated cells a complete restoration of the response occurred.

6.3.2.3 Collaboration across the membrane in both directions

If the partial restoration of the anti-DNP response was via a soluble factor, it would be expected that the collaboration would operate in both directions. Therefore, the system was reversed and the anti-θ treated spleen population cultured in the upper chamber and untreated spleen or purified T or anti-θ treated (spleen cells) cultured in the lower chamber. In this case, even better restorations,

Table 6.2 : Transfer of a T cell replacing factor across a Millipore membrane

Cells Cultured [a]		Anti-DNP PFC response in lower chamber [b]				
Upper chamber	Lower chamber	1.	2.	3.	4.	5.
Untreated	Untreated	650±80	1143±148	1210±150	1733±100	2347±101
-	Anti-θ treated	165±18	233±13	190±40	295±89	653±24
Untreated	Anti-θ treated	615±102	563±37	695±157	690±34	1845±238

a) 5×10^6 viable cells were cultured in 1 ml in the upper chamber and 5×10^6 in 2 ml in the lower chamber.

b) Each value represents the arithmetic mean of four cultures ± the standard error of the mean.

Table 6.3 : The effect of culturing fractionated cells in the upper chamber on production of the factor.

Cells Cultured [a]		Anti-DNP PFC response in lower chamber [d]			
Upper Chamber	Lower Chamber	1.	2.	3.	4.
Untreated	Untreated	728±123	1343±83	1780±83	1578±116
- [b]	Anti-θ treated	143±29	295±16	440±18	485±54
Untreated	Anti-θ treated	335±59	515±33	933±175	830±51
T [c]	Anti-θ treated	n.d. [e]	335±52	413±113	n.d.
-	Anti-θ treated + T	635±56	1420±20	1380±170	n.d.
Anti-θ treated	Anti-θ treated	n.d.	318±27	n.d.	506±9
-	T	n.d.	O	<5	n.d.

a) 5×10^6 viable cells were cultured in 1 ml in the upper chamber and 5×10^6 cells in 2 ml in the lower chamber.

b) No cells.

c) T cells = upper layer from Isopaque/Ficoll separation of Ig-bearing and θ-positive lymphocytes.

d) Each value represents the arithmetic mean of four cultures ± the standard error of the mean.

e) Not done.

averaging 55% and ranging between 40 and 70% in individual experiments, were obtained (Table 6.4).

This result probably reflected the geometry of the culture vessel. Cells in the lower chamber gave consistently higher responses (>2 fold) to DNP-MON than the same number of cells cultured in the upper chamber, and possibly therefore, more factor was produced by cells cultured in the lower chamber. Again there was no restoration by purified T cells or B cells cultured alone.

6.3.2.4 Effect of removal of the Ig-bearing T cell on production of the collaborative factor

As mentioned earlier (6.3.1) Ig bearing T cells sediment to the lower layer in the Isopaque/Ficoll separation procedure, and one possibility was that these cells, which can act as functional helper cells (Table 6.1), were responsible for the production of the collaborative factor.

To investigate this possibility the upper layer T cells (i.e. depleted of the Ig positive T cells) were added to anti-θ treated cells, cultured in the upper chamber, and the lower chamber response measured (Table 6.5). The collaborative factor was once again produced indicating that the Ig bearing T cell is not essential for factor production.

6.3.3 Properties of the collaborative factor

6.3.3.1 Inability of the factor to pass through a dialysis membrane

Normal spleen cells were cultured in the upper chamber and were separated from anti-θ treated spleen cells in the lower chamber by a dialysis membrane, rather than a Millipore membrane. DNP-MON was present in both chambers. Under these culture conditions no restoration of the response of the lower chamber cells was obtained (Table 6.6). From this result it was concluded that the collaborative factor was non-dialyzable i.e. has a molecular weight >5000-10000.

6.3.3.2 Specificity

The specificity of the factor for the antigen DNP-MON was investigated using another T dependent response, the response to SRBC.

Table 6.4 : Restoration of response in upper chamber by factor production in the lower chamber.

Cells Cultured [a]		Anti-DNP PFC in upper chamber [c]		
Upper Chamber	Lower Chamber	1.	2.	3.
Untreated	Untreated	663±48	285±50	734±38
Anti-θ treated	-	141±27	47±18	293±21
Anti-θ treated	Untreated	630±90	258±55	545±86
Anti-θ treated	T [b]	n.d. [d]	n.d.	240±20
Anti-θ treated + T	-	n.d.	n.d.	608±77
T alone	-	n.d.	n.d.	<10
Anti-θ treated	Anti-θ treated	n.d.	n.d.	150±38

a) 5×10^6_6 viable cells were cultured in 1 ml in the upper chamber and 5×10^6 cells in 2 ml in the lower chamber.

b) T cells = upper layer from Isopaque/Ficoll separation of Ig-bearing and θ-positive lymphocytes.

c) Each value represents the arithmetic mean of four cultures ± the standard error of the mean.

d) Not done.

Table 6.5 : Effect of removal of Ig-bearing T cells on production of the factor.

Cells Cultured [a]		Anti-DNP PFC [d] in
Upper chamber	Lower chamber	Lower chamber
Untreated	Untreated	1695±95
- [b]	Anti-θ treated	655±93
Untreated	Anti-θ treated	1375±93
T [c]	Anti-θ treated	580±91
-	Anti-θ treated + T	1387±105
-	T	<5
Anti-θ treated	Anti-θ treated	815±60
Anti-θ treated + T	Anti-θ treated	1227±125

a) 5×10^6 viable cells of each type were cultured in 1 ml in the upper chamber and 5×10^6 cells of each type in 2 ml in the lower chamber.

b) No cells.

c) T cells = upper layer from Isopaque/Ficoll separation of Ig-bearing and θ-positive lymphocytes.

d) Each value represents the arithmetic mean of four cultures ± the standard error of the mean.

Table 6.6 : Inability of factor to cross a dialysis membrane.

Cells Cultured [a]		Anti-DNP PFC in Lower chamber [d]	
Upper chamber	Lower chamber	1.	1. 2.
Untreated	Untreated	1768±54	1150±87
- [b]	Anti-θ treated	427±22	228±18
Untreated	Anti-θ treated	436±63	398±53
-	Anti-θ treated + T [c]	n.d. [e]	1405±121

a) 5×10^6 viable cells were cultured in 1 ml in the upper chamber, 5×10^6 cells in 2 mls in the lower chamber. Upper and lower chambers were separated by a dialysis membrane.

b) No cells.

c) T cells = upper layer from Isopaque/Ficoll separation of Ig bearing and θ positive lymphocytes.

d) Each value is the arithmetic mean of four cultures ± the standard error of the mean.

e) Not done.

Table 6.7 : Specificity of factor for the DNP-MON response.

Cells Cultured [a]		Antigen [c]		PFC response in lower chamber [d]	
Upper chamber	Lower chamber	Upper chamber	Lower chamber	Anti-DNP PFC	Anti-SRBC PFC
Untreated	Untreated	DNP-MON SRBC	DNP-MON SRBC	973±132	1078±255
-[b]	Anti-θ treated	DNP-MON SRBC	DNP-MON SRBC	180±21	10±6
Untreated	Anti-θ treated	DNP-MON SRBC	DNP-MON SRBC	810±49	108±17
Untreated	Anti-θ treated	DNP-MON	DNP-MON SRBC	540±72	10±6
Untreated	Anti-θ treated	SRBC	SRBC	-	78±31

a) 5×10^6 viable cells were cultured in 1 ml in the upper chamber and 5×10^6 cells in 2 ml in the lower chamber.

b) No cells.

c) DNP-MON was at a final concentration of 100ng/ml and sterile washed CSL SRBC at 5×10^6 cells/ml.

d) Each value represents the arithmetic mean of four cultures ± the standard error of the mean. Cultures were assayed on day 4.

Spleen cells primed 7 weeks previously to SRBC were used so that good *in vitro* anti-SRBC responses were obtained. These cells were cultured in the upper chamber with both antigens (SRBC and DNP-MON) or either antigen alone and the lower chamber anti-DNP and anti-SRBC responses measured. Cultures were assayed on day 4, instead of day 3 as previously, as the anti-SRBC response was highest at this time. Results of one of a number of similar experiments are given in Table 6.7.

Whilst restoration of the anti-DNP response was around 45%, no restoration of the anti-SRBC response was obtained when DNP-MON alone was the antigen present in the upper chamber, indicating that the factor was not simply mitogenic for B cells in the lower chamber but was antigen specific.

Slight but statistically significant restorations of the SRBC response (6-9%) were obtained when SRBC were present in the upper chamber, suggesting that a specific SRBC collaborative factor might also be produced. Restoration of the anti-SRBC response might not be expected to be as great as for DNP-MON as a SRBC-factor complex would be unable to cross the Millipore membrane.

6.4 DISCUSSION

In the experiments reported in this chapter, the ability of T cells to collaborate with B cells across a cell impermeable Millipore membrane was assessed. It was shown that purified T cells were unable to collaborate with B cells in the anti-hapten response when these cells were separated by a Millipore membrane. They were, though, excellent helpers when cultured together with the B cells below the membrane. If both T and B cells were cultured above the membrane, however, a collaborative factor was produced which was partially able to restore the anti-DNP response of B cells cultured below. B cells alone could not produce the factor.

The possibility of cell leakage through the Millipore membrane seemed unlikely for several reasons.

a) The Millipore membranes were attached by siliconized rubber rings which were well beyond the medium inter-

face. Hence, if the membrane was intact no leakage should have occurred. Millipore membranes were examined when cells were harvested for any cracks and defective chambers were discarded.

b) The response to DNP-MON is so highly T-dependent that the failure of T cells to give any help at all across the membrane was a good positive control against cell leakage.

c) The collaborative factor was shown to work in both directions, i.e. could diffuse up as well as down and to some extent this also argued against cell leakage through the membrane, as during culture cells very rapidly settled to the bottom of the chambers.

The factor was shown to be specific and non-dialyzable. These two properties are reminiscent of the factor described by Feldmann and Basten (1972b, c, d) which was obtained from ATC. It is of interest, therefore, that in our system T cells alone cannot give rise to this factor. Whether the factor was a T or B cell product could not at this stage be concluded. The possibility that immunoglobulin bearing T cells were necessary for its production was eliminated, however, by the ability of purified T cells (i.e. depleted of Ig bearing cells) and anti-θ treated spleen (B cells) to still produce the factor. In the next chapter it will be shown that the flagellin specific B cell has a role in the anti-hapten response to DNP-MON and in particular appears to be necessary for production of the collaborative factor.

6.5 SUMMARY

The *in vitro* anti-hapten response to DNP-flagellin (DNP-MON) was used to examine the ability of T cells to collaborate with B cells across a cell impermeable Millipore membrane. Pure populations of T and B lymphocytes were obtained by the Isopaque/Ficoll separation procedure. It was found that T cells alone were not able to collaborate with B cells across a Millipore membrane. However, B and T cells together produced a T cell replacing factor which passed through the membrane and partially restored the antibody

response of B cells cultured beneath the membrane. The T cell replacing factor was shown to be non-dialyzable and antigen specific.

CHAPTER 7

A role for the carrier specific B cell and macrophages in cellular collaboration

7.1 INTRODUCTION

In Chapter 6, it was shown that when either T or B cells were cultured alone above a cell-impermeable Millipore membrane, they were unable to restore the anti-DNP response of B cells cultured below the membrane. B and T cells together however, produced a factor which passed through the Millipore membrane and which partially restored the anti-DNP response of the B cells below the membrane. This factor was non-dialyzable and specific. In this chapter production of the collaborative factor is further analyzed and, in particular, the role played by carrier specific B cells and macrophages in factor production considered.

7.2 MATERIALS AND METHODS

7.2.1 Animals, antigens, tissue culture and assay procedures

were as described in the previous chapter (6.2) and in Chapter 3 (3.2).

7.2.2 Irradiation of mice

Mice received 850 rads whole body gamma radiation from a ^{60}Co source over a period of 20-25 minutes. The mice were housed (5 per container) in perspex cylindrical containers which rotated around the source as well as around their own axis during irradiation.

7.2.3 Irradiation of cells

Cells were exposed in capped, plastic centrifuge tubes (2.5×10^7 viable cells in 1.0 ml F15 medium) to the gamma radiation of the ^{60}Co source at the rate of 245 rads per minute. The cells were rotated, like the mice, both around their own axis and around the source during exposure to the radiation.

7.2.4 Titration of anti-POL antibody

POL specific antibody was detected by a haemagglutination procedure described in detail by Langman (1972). Briefly, SRBC or HRBC were coated with POL, via $CrCl_3$, and added to dilutions of the serum to be tested in V-cupped perspex trays. The serum/SRBC mixtures were allowed to settle for 4-6 h at room temperature before 50% agglutination was taken as the endpoint of each titration.

7.2.5 Preparation of POL specific rosettes and depletion on Isopaque/Ficoll

Washed SRBC were coated with POL 870 as follows. To 4.0 ml of normal saline 0.25 ml of packed SRBC were added followed by 100 µl (1 mg) of POL 870 and 200 µl of 0.1% (w/v) $CrCl_3$ in normal saline. All were mixed well and left for 10 minutes at room temperature. PBS (5 ml) was then added and the red cells centrifuged (300 g, 5 min) resuspended in 5 ml PBS and again washed. The red cell pellet was finally resuspended in 1.0 ml of F15 medium to give 20% E-POL cells for rosetting. Rosettes were formed as described in 5.2.4.1 and separated on the Isopaque/Ficoll mixture as usual (5.2.5). A 20% suspension of unsensitized SRBC (E) was used for a control rosetted population.

7.2.6 Radioiodination of POL

POL was iodinated for the suicide experiments with carrier-free iodide -125 (IMS 3 Radiochemical Centre, Amershan, U.K.) by a direct oxidation procedure (Byrt and Ada, 1969). Preparations of ^{125}I-POL had a specific activity of 50-120 µC/µg. POL was also iodinated with the non-radioactive iodide-127 by the same procedure, for use as a control.

7.2.7 Suicide of spleen cells with ^{125}I-POL

Cells were incubated at a concentration of 2.5×10^7 -1×10^8/ml in culture medium containing 15mM sodium azide and 5-20µg/ml ^{125}I-POL for 1h at 37°C. They were then washed several times before being counted and subsequently cultured. Control suicided cells were treated identically except that ^{127}I-POL was added. For some of the *in vivo* transfers, cells were left for 14-19 h at 4°C after the washing stage before being injected into irradiated mice according to the method of Cooper and Ada (1972).

7.2.8 Removal of Macrophages

7.2.8.1 By adherence

Spleen cells were cultured in F15 with 10% FCS for 5h at 37°C at monolayer density in plastic tissue culture dishes. This procedure allowed macrophages to adhere and spread out on the plastic surface. Most of the non-adherent cells were then harvested by gentle pipetting which left the

adherent cells on the plastic. The efficiency of this procedure at depleting macrophages has been described elsewhere (Gardner et al., 1974a).

7.2.8.2 By carbonyl iron method

To 5 ml of spleen cells (2×10^7 cells/ml) in a screw capped McCartney bottle sterile, carbonyl iron powder was added until the medium was grey in colour. The bottle was sealed tightly and rotated at 37°C for 30 min. The cells were then transferred into a sterile glass centrifuge tube and a strong magnet run down the sides of the tube several times. The supernatant, containing most of the non-phagocytic cells was removed and the filings in the bottom of the tube were resuspended in a further volume of medium and the procedure repeated. Cells recovered in this way were washed and depleted of carbonyl iron in the same way (usually 3 times) until no grey colour was apparent. At least 80% of cells were recovered following this procedure and most dead cells were removed, the recovered cells generally having viabilities >95% as judged by trypan blue dye exclusion.

7.3 RESULTS

7.3.1 The effect of removal of the POL specific B cell on the anti-hapten response to DNP-MON in vitro

In these studies two methods were used to remove POL specific cells from spleen cell populations. In the first method POL specific cells were "suicided" with radioactive antigen. In the second procedure POL cells were first rosetted with POL coated red cells and then separated from the non-rosetting spleen cells by centrifugation on Isopaque/Ficoll. Before using radioactive POL to suicide specific cells the radiation sensitivity of the in vitro anti-DNP response to DNP-MON was determined.

7.3.1.1 The effect of γ-irradiation on the anti-DNP response to DNP-MON in vitro

Initially spleen cells were irradiated by exposure to a ^{60}Co source for varying time intervals. Cells received doses between 250 and 3675 rads of irradiation prior to

culture *in vitro* with DNP-MON. It was found that the anti-DNP response was highly radiosensitive, radiation doses of ≥ 1000 rads almost completely obliterating the anti-DNP response (Figure 7.1).

The radiosensitivity of the T (helper) cells in this system was then determined. Splenic T cells were purified by the Isopaque/Ficoll procedure and added to anti-θ treated spleen cells. Prior to addition to the anti-θ treated spleen the T cells were exposed to irradiation doses ranging from 600 to 3675 rads (Table 7.1).

Even after very high doses of irradiation the T cells were still able to bring about significant restoration (> 40%) of the anti-DNP response. There thus appeared to be a radiation sensitive T cell component and a radiation resistant component. These findings are consistent with those of others (Kettman and Dutton, 1971; Vann and Kettman, 1972; Hamaoka et al., 1972), and probably reflect the degree of differentiation of the helper cells e.g. whether they have to divide or not. From Figure 7.1, however, it appeared likely that most B cells are damaged by irradiation doses greater than about 700 rads. Thus any decrease in the anti-DNP-MON response following POL suicide could be due to either inactivation of POL specific B cells or elimination of the radiosensitive T cells.

7.3.1.2 The effect of ^{125}I-POL suicide on the anti-DNP response to DNP-MON

When spleen cells were reacted with ^{125}I-POL of high specific activity (37°C, 1h) prior to cell culture with DNP-MON, a decrease in the anti-DNP PFC response was consistently observed. In 9 similar experiments, the average decrease was 53% and ranged between 40 and 74% in individual experiments. Some typical results are summarized in Table 7.2.

7.3.1.3 The specificity of the ^{125}I-POL suicide *in vitro*

To investigate the specificity of the POL suicide, spleen cells from mice which had been primed 3 days previously with 2×10^5 ORBC, a procedure known to prime helper T cells preferentially for an anti-SRBC response (Cunningham and Sercarz, 1971), were suicided as usual with POL and

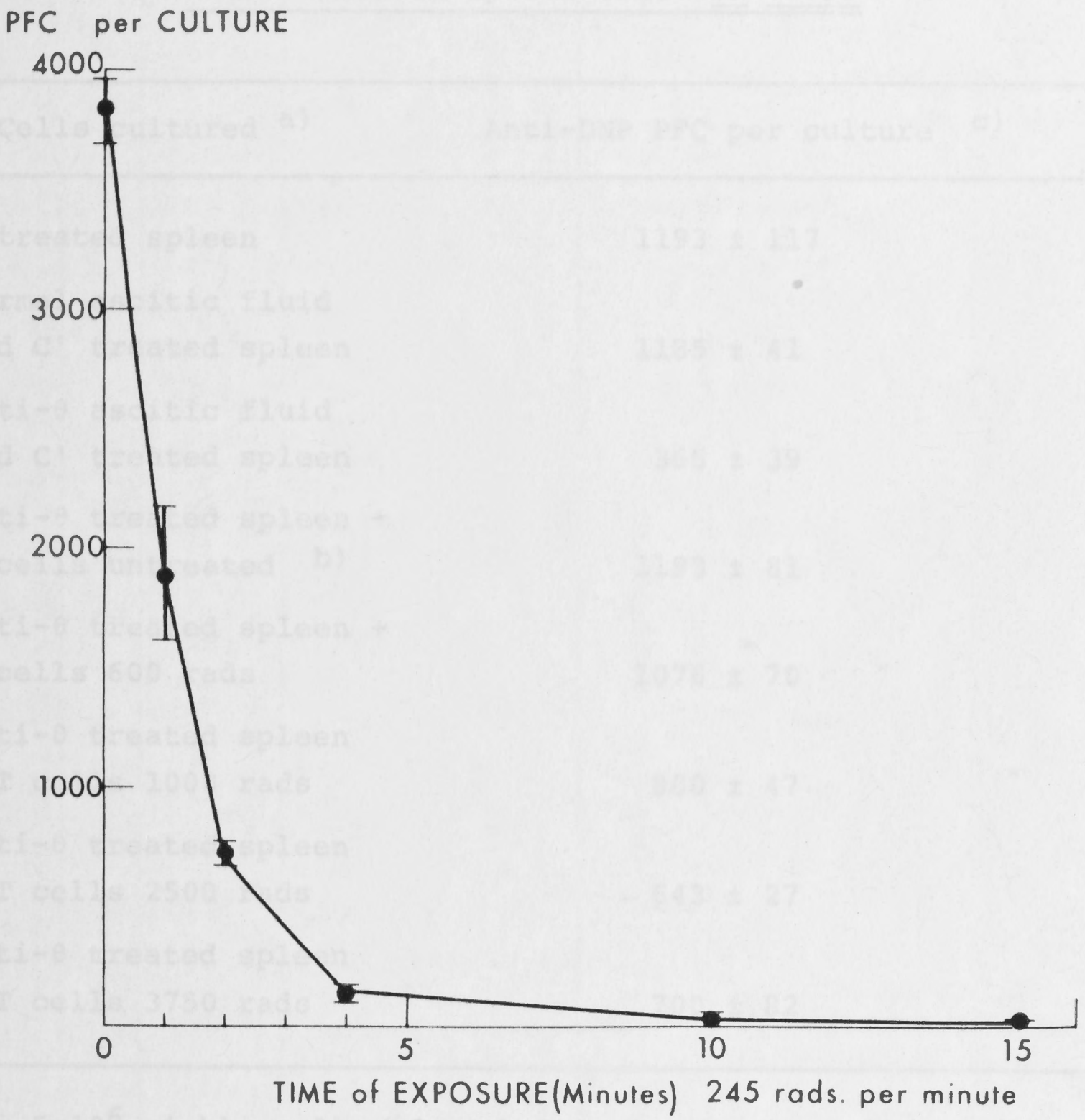


Figure 7.1 The effect of ^{60}Co irradiation of spleen cells on their anti-DNP response to DNP-MON. Approximately $5x10^6$ cells were cultured per well. Each point represents the arithmetic mean of four cultures ± the standard error of the mean.

Table 7.1: The effect of radiation of T cells on their ability to restore the anti-DNP response of anti-θ treated spleen cells in vitro

Cells cultured [a]	Anti-DNP PFC per culture [c]
Untreated spleen	1193 ± 117
Normal ascitic fluid and C' treated spleen	1185 ± 41
Anti-θ ascitic fluid and C' treated spleen	365 ± 39
Anti-θ treated spleen + T cells untreated [b]	1193 ± 81
Anti-θ treated spleen + T cells 600 rads	1076 ± 70
Anti-θ treated spleen + T cells 1000 rads	800 ± 47
Anti-θ treated spleen + T cells 2500 rads	643 ± 27
Anti-θ treated spleen + T cells 3750 rads	700 ± 82

a) 5×10^6 viable cells of each type were cultured per well

b) T cells = upper layer from Isopaque/Ficoll separation of Ig-bearing and θ-positive lymphocytes.

c) Each value is the arithmetic mean of 4 cultures ± the standard error of the mean.

Table 7.2: The effect of ^{125}I-POL suicide on the anti-DNP response to DNP-MON in vitro

Cells cultured [a]	Anti-DNP PFC per culture [b]			
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
Untreated	1198 ± 98	1228 ± 31	1440 ± 61	1935 ± 96
^{125}I-POL treated	648 ± 118	588 ± 14	758 ± 61	830 ± 101
^{127}I-POL treated	1298 ± 33	1075 ± 108	1238 ± 112	1628 ± 27

a) $5x10^6$ viable cells were cultured per well. DNP-MON at 100ng/ml.

b) Each value represents the arithmetic mean of 4 wells ± the standard error of the mean.

cultured with both DNP-MON and SRBC. Cultures were assayed on day 3 against both DNP coated SRBC and normal SRBC so that the response to both antigens in the same culture was measured (Table 7.3).

It was shown that the suicide was specific for the anti-DNP response as the anti-SRBC response was the same in both the POL suicided (^{125}I-POL) and control (^{127}I-POL) reacted cell populations.

7.3.1.4 The specificity of the ^{125}I-POL suicide *in vivo*

Some of the suicided cells were transferred into irradiated recipients which were challenged on days 2 and 5 with 10 µg POL and $2x10^8$ SRBC, intravenously. Serum was collected and spleens harvested on day 8 after cell transfer and the anti-POL haemagglutination titre and anti-SRBC PFC response measured. Although the anti-POL antibody titre was significantly reduced or abolished following ^{125}I-POL suicide, the anti-SRBC PFC response was only slightly affected, if at all, by the suicide procedure (Table 7.4). In two other experiments the POL antibody response was only partially reduced. These *in vivo* results showed conclusively that the POL specific B cell was eliminated by the POL suicide procedure, as the antibody response to POL is T independent (Feldmann and Basten, 1971). It was not known however, whether some POL specific T cells were also affected.

7.3.1.5 The cell affected by ^{125}I-POL suicide *in vitro*

To investigate further the cell affected by the suicide procedure, suicided spleen cells were fractionated on Isopaque/Ficoll prior to culture. In some experiments spleen cells from low dose ORBC primed mice were used, so that a specificity control could be included as previously (7.3.1.3).

The ability of T cells from suicided spleen to restore the response of anti-θ treated spleen cells was compared with the restorative capacity of both control suicided and untreated T cells. In addition, the ability of B cells from either suicided spleen or control spleen to respond when mixed with normal T cells was determined. It can be seen (Table 7.5) that the T cell compartment appeared to be

Table 7.3: The specificity of ^{125}I-POL suicide for the DNP-MON response in vitro

Cells cultured [a]	Anti-DNP PFC per culture [b]	Anti-SRBC PFC per culture [b]
Untreated	2043 ± 96	638 ± 46
^{125}I-POL treated	543 ± 69	463 ± 57
^{127}I-POL treated	1783 ± 151	415 ± 28

a) $5x10^6$ viable cells were cultured per well. Mice had received $2x10^5$ ORBC 3 days prior to culture.

DNP-MON was at a final concentration of 100ng/ml and sterile, washed CSL SRBC were at $5x10^6$/ml.

b) Each value represents the arithmetic mean of 4 wells ± the standard error of the mean.

Anti-DNP plaques were obtained by subtracting the anti-SRBC plaques from the total number of plaques formed on DNP-coated SRBC.

Table 7.4: The specificity of ^{125}I-POL suicide in vivo

Cells transferred [a]	Anti-POL haemagglutination titre [b]	Anti-SRBC PFC per spleen [c]
Untreated	4.4 ± 0.2	26560 ± 3422
^{125}I-POL	<0.5	27520 ± 3759
^{127}I-POL	4.3 ± 0.3	25920 ± 2080
No cells	<0.5	70 ± 45

a) Each mouse received 10^7 viable cells.

b) The haemagglutination titre was judged as the last well showing positive haemagglutination. Well 1 contained a 1/5 dilution of antiserum. Each value is the arithmetic mean of 5 serum titrations ± the standard error of the mean.

c) Each value is the arithmetic mean of 5 spleen ± the standard error of the mean.

Table 7.5 : The cell affected by ^{125}I-POL suicide in vitro

Cells cultured [a) b)]	PFC per culture		
	Anti-DNP		Anti-SRBC
	Expt. 1	Expt. 2	Expt. 1
Untreated spleen	2615 ± 123	2028 ± 93	1040 ± 93
Anti-θ treated spleen	395 ± 28	443 ± 44	50 ± 13
Anti-θ treated spleen + T cells untreated	2215 ± 188	1118 ± 228	1560 ± 82
Anti-θ treated spleen + T cells ^{125}I-POL treated	2320 ± 95	1054 ± 71	1565 ± 150
Anti-θ treated spleen + T cells ^{127}I-POL treated	1895 ± 186	1224 ± 44	1560 ± 93
^{125}I-POL treated B cells	30 ± 10	n.d. [d)]	n.d.
^{125}I-POL treated B cells [c)] + T cells untreated	260 ± 43	n.d.	125 ± 13
^{127}I-POL treated B cells	240 ± 52	n.d.	n.d.
^{127}I-POL treated B cells + T cells untreated	1010 ± 80	n.d.	185 ± 40

a) In experiment 1, $5x10^6$ viable cells of each type were cultured. DNP-MON was at a final concentration of 100ng/ml and SRBC at $5x10^6$/ml.

b) In experiment 2, $5x10^6$ untreated and anti-θ treated cells and $1x10^6$ T cells were cultured.

c) B cells = lower layer from Isopaque/Ficoll separation of Ig-bearing and θ-positive lymphocytes. T cells = upper layer from same separation.

d) Not done.

completely intact following the suicide procedure. T cells isolated from the suicided spleen were as good as T cells from the control suicided and untreated spleen at restoring both the anti-DNP and anti-SRBC responses of anti-θ treated spleen. The lesion in the response was in the lower layer (B cells), the suicided population giving a significantly lower anti-DNP response than the control population.

The anti-SRBC response of these lower layer cells was rather disappointing, however. B cells were recovered by lysis of the SRBC used in the rosetting procedure, and residual SRBC and SRBC stroma caused interference of the anti-SRBC response of these B cells. Even so, there was no indication of a non-specific decrease in the anti-SRBC response following POL suicide.

7.3.1.6 The cell affected by ^{125}I-POL suicide *in vivo*

The same experiment as that reported in 7.3.1.5 was carried out *in vivo*. Following ^{125}I-POL suicide of normal or DNP-BSA primed spleen cells, the cells were fractionated on Isopaque/Ficoll, transferred into irradiated recipients and challenged with 5 μg of DNP-MON intravenously. Six days later spleens were harvested and anti-DNP PFC measured. The response of normal spleen cells is shown in Table 7.6.

Suicide with ^{125}I-POL reduced the *in vivo* direct PFC response to DNP by about the same extent as the *in vitro* response (55% reduction). T cells from the suicided and control treated spleen cells were equally good at restoring the anti-DNP PFC response showing again that the T cell function was unaffected by the suicide procedure. It is of interest that the T cells from the suicided population apparently homed to the spleen unimpeded. The lesion again appeared in the B cell compartment, the degree of depression of the response being of the same order as for the whole spleen (54% reduction).

A similar reduction in the direct PFC response to DNP was obtained when DNP-BSA primed spleen cells were suicided with ^{125}I-POL. In addition, suicide reduced the indirect PFC response even more dramatically (85 -90% reduction). As with the primary response, the suicide procedure inactivated B cells and not T cells (Table 7.7).

Table 7.6: The effect of ^{125}I-POL suicide on the *in vivo* response of normal spleen cells to DNP-MON

Cells transferred [a]	Direct anti-DNP PFC per spleen [d]
No cells	64 ± 10
Untreated spleen	3504 ± 566
^{125}I-POL treated spleen	1550 ± 307
^{127}I-POL treated spleen	2952 ± 228
Anti-θ treated spleen	298 ± 150
Anti-θ treated spleen [b] + T cells ^{125}I-POL treated	1888 ± 298
Anti-θ treated spleen [b] + T cells ^{127}I-POL treated	1924 ± 142
^{125}I-POL treated B cells + T cells untreated [c]	1024 ± 107
^{127}I-POL treated B cells [c] + T cells untreated	2256 ± 200

a) 10^7 viable cells were injected into each mouse.

b) $7x10^6$ anti-θ treated cells and $3x10^6$ T cells were injected into each mouse.

c) $7x10^6$ B cells and $3x10^6$ T cells were injected into each mouse. B cells = lower layer from Isopaque/Ficoll separation of Ig-bearing and θ-positive lymphocytes. T cells = upper layer from same separation.

d) Each value represents the arithmetic mean of five spleens ± the standard error of the mean. Only the direct PFC response is quoted as little or no indirect PFC were detected.

Table 7.7 : The effect of ^{125}I-POL suicide on the *in vivo* response of DNP-BSA primed spleen cells to DNP-MON

Cells transferred [a]	Anti-DNP PFC per spleen [d] Direct	Indirect
No cells	63 ± 13	<5
Untreated spleen	3106 ± 400	1635 ± 290
^{125}I-POL treated spleen	1136 ± 280	164 ± 110
^{127}I-POL treated spleen	2608 ± 430	1716 ± 342
Anti-θ treated spleen + T cells ^{125}I-POL treated [b]	2756 ± 528	1732 ± 228
Anti-θ treated spleen + T cells ^{127}I-POL treated [b]	2780 ± 276	1650 ± 430
^{125}I-POL treated B cells + T cells untreated [c]	792 ± 90	368 ± 214
^{127}I-POL treated B cells + T cells untreated [c]	1935 ± 488	2075 ± 458

a) 10^7 viable DNP-BSA primed cells were injected into each mouse.

b) $7x10^6$ anti-θ treated cells and $3x10^6$ T cells were injected into each mouse.

c) $7x10^6$ B cells and $3x10^6$ T cells were injected into each mouse. B cells = lower layer from Isopaque/Ficoll separation of Ig-bearing and θ-positive lymphocytes. T cells = upper layer from same separation.

d) Each value represents the arithmetic mean of five spleens ± the standard error of the mean.

7.3.1.7 The effect of removal of POL rosette-forming cells on the anti-DNP response to DNP-MON

The decrease in the anti-DNP response following removal of the POL specific B cell by ^{125}I-POL suicide was confirmed by another method of depletion, removal of POL specific rosette forming cells on Isopaque/Ficoll. SRBC were coated with POL, via $CrCl_3$, POL specific rosettes formed and this rosetted cell suspension spun on Isopaque/Ficoll as for depletion of Ig RFC. The POL specific rosettes sank to the lower layer and the remaining cells floated (upper layer). These upper layer cells, control rosetted upper layer cells (i.e. cells reacted with normal SRBC before centrifugation) and untreated spleen cells were cultured with DNP-MON as usual.

A decrease similar to that observed in the ^{125}I-POL suicided population was observed in spleen cells depleted of POL RFC (Table 7.8). This result is consistent with the rosetting procedure removing POL specific B cells. Furthermore, other workers have demonstrated that antigen specific rosettes are preferentially formed by B cells and not by helper cells (Elliott et al., 1973). The decrease in responsiveness was more variable following rosette depletion than following suicide, this result probably reflecting the lower efficiency of the rosetting procedure at removing POL specific B cells.

7.3.2 The effect of removal of the POL specific B cells by ^{125}I-POL suicide on production of the specific T cell replacing factor

Removal of POL specific B cells produced a decrease in the response to DNP-MON of the order of 50%. Similarly, T and B cells cultured above a cell impermeable Millipore membrane produced a factor which passed through the membrane and restored the anti-DNP response of B cells below the membrane by approximately 50% (Chapter 6).

It was thought, therefore, that this restoration might represent the B cell contribution to the anti-DNP response. Hence the effect of ^{125}I-POL suicide on production of the collaborative factor was examined. Suicided spleen and control suicided spleen cells were cultured above the Millipore membrane and the anti-DNP response of anti-θ treated

Table 7.8: The effect of removal of POL rosette-forming cells on the anti-DNP response to DNP-MON in vitro

Cells cultured [a]	Anti-DNP PFC per culture	
	Expt. 1 [b]	Expt. 2 [c]
Untreated	860 ± 111	1525 ± 115
POL rosette depleted	450 ± 128	658 ± 62
Control rosette depleted	720 ± 47	1398 ± 80

a) 5×10^6 viable cells were cultured per well.

b) Each value is the arithmetic mean of 4 wells ± the standard error of the mean.

c) Each value is the arithmetic mean of 8 wells ± the standard error of the mean.

spleen cells (i.e. B cells), cultured below, measured. It can be seen that ^{125}I-POL suicide abolished production of the collaborative factor (Table 7.9), highlighting the importance of the POL specific B cell in allowing collaboration to occur across a Millipore membrane.

7.3.3 The role of the macrophage in the response to DNP-MON in vitro

Having established the importance of the carrier specific B cell in production of the collaborative factor, it was of some interest to investigate the means by which the factor acted on B cells in the lower chamber. In particular, do macrophages play a role as has been reported for other specific T cell replacing factors (Feldmann, 1972c; Gisler et al., 1973).

7.3.3.1 The ability of macrophage depleted spleen cells to respond to DNP-MON

Initially, the effect of removal of macrophages on the ability of spleen cells to respond to DNP-MON was investigated. It was shown that when macrophages were removed by either of two procedures - an adherence method or a procedure utilizing carbonyl iron powder - a decrease of 40-50% in the anti-DNP PFC response was observed (Table 7.10). This decrease corresponded to the carrier specific B cell contribution to the response (see 7.3.1.5).

7.3.3.2 The role of macrophages in both the production and action of the collaborative factor

When macrophage depleted spleen populations (carbonyl iron procedure) were cultured above a Millipore membrane restoration of the anti-DNP response of B cells cultured below was virtually abolished, indicating that macrophages play a role in production of the factor (Table 7.11).

In contrast, it appears that the action of the collaborative factor is macrophage independent as it was found that the factor restored the response of anti-θ treated spleen depleted of macrophages (Table 7.11). It should be noted, however, that macrophage depleted anti-θ treated spleen gave a lower response than undepleted anti-θ treated spleen.

Table 7.9: The effect of ^{125}I-POL suicide on production of the collaborative factor

Cells cultured [a]		Anti-DNP response in lower chamber [b]	
Upper chamber	Lower chamber	Expt. 1	Expt. 2
Untreated	Untreated	2625 ± 264	1515 ± 207
-	Anti-θ treated	500 ± 41	435 ± 57
Untreated	Anti-θ treated	895 ± 123	1125 ± 390
^{125}I-POL treated	Anti-θ treated	350 ± 66	423 ± 117
^{127}I-POL treated	Anti-θ treated	1100 ± 114	n.d. [c]
-	^{125}I-POL treated	1287 ± 98	n.d.
-	^{127}I-POL treated	2290 ± 158	n.d.

a) $5x10^6$ viable cells were cultured in 1ml in the upper chamber and $5x10^6$ in 2ml in the lower chamber.

b) Each value represents the arithmetic mean of 4 cultures ± the standard error of the mean.

c) Not done.

Table 7.10: The effect of removal of macrophages on the anti-DNP response to DNP-MON *in vitro*

Cells cultured [a]	Anti-DNP PFC per culture [b]		
	Expt. 1	Expt. 2	Expt. 3
Untreated	1168 ± 59	3029 ± 250	1295 ± 208
Macrophage depleted (adherence method)	705 ± 122	n.d. [c]	n.d.
Macrophage depleted (carbonyl iron method)	n.d.	1691 ± 89	653 ± 93

a) $5x10^6$ viable cells were cultured per well.

b) Each value represents the arithmetic mean of 4 cultures ± standard error of the mean.

c) Not done.

Table 7.11 : The effect of removal of macrophages on factor production and the restoration of the anti-DNP response

Cells cultured [a] Upper chamber	Lower chamber	Anti-DNP PFC in Lower chamber [c] Expt. 1	Expt. 2
Untreated	Untreated	3155 ± 232	1515 ± 207
- [b]	Anti-θ treated	970 ± 54	435 ± 57
Anti-θ treated	Anti-θ treated	1013 ± 18	555 ± 152
Untreated	Anti-θ treated	1660 ± 102	1125 ± 390
Untreated (-macrophages)	Anti-θ treated	1073 ± 103	605 ± 64
Untreated	Anti-θ treated (-macrophages)	1147 ± 110	645 ± 83
-	Anti-θ treated (-macrophages)	420 ± 53	160 ± 83
-	Anti-θ treated + T [c]	2700 ± 140	n.d. [d]
Anti-θ treated	Anti-θ treated (-macrophages)	n.d.	190 ± 71

a) 5×10^6 viable cells were cultured in 1ml in the upper chamber and 5×10^6 in 2ml in the lower chamber.

b) No cells.

c) T cells = upper layer from Isopaque/Ficoll separation of Ig-bearing and θ-positive lymphocytes.

d) Not done.

e) Each value is the arithmetic mean of 4 cultures ± the standard error of the mean.

Such a result suggests that macrophages can aid direct triggering of B cells by antigen. However, the anti-DNP response of anti-θ treated spleen was restored to a similar extent (approx. 25-30% of the total anti-DNP response of untreated spleen in these experiments) whether macrophages were present or absent.

The possibility that macrophages in the upper chamber supplied a growth requirement to the anti-θ treated, macrophage deficient, population in the lower chamber was eliminated as macrophages (i.e. anti-θ treated spleen cells) in the upper chamber were unable to reconstitute the lower chamber response. Collectively these results suggest that a non-phagocytic cell (e.g. K cell) may be able to utilize the collaborative factor to stimulate B cells, or the factor and antigen may stimulate B cells directly.

7.4 DISCUSSION

The results reported in this chapter have established a role for the carrier specific B cell in the induction of an anti-hapten response to DNP-MON both *in vivo* and *in vitro*. It was shown that removal of the carrier specific B cell by ^{125}I-POL suicide decreased the anti-DNP response of spleen cells by 40-50%. Furthermore, following suicide of POL specific B cells spleen cells were unable to produce a specific T cell replacing factor which could pass through a cell-impermeable Millipore membrane and partially restore the anti-DNP response of B cells below the membrane. From these results it was concluded that production of the collaborative factor which passed through the Millipore membrane represented the contribution of carrier specific B cells to the anti-DNP response.

The validity of the above conclusion rests on the fact that it was the B cell that was destroyed by the suicide procedure. This appeared to be the case, as:-

a) B cells were much more sensitive to radiation damage than T cells, some helper T activity remaining after doses of irradiation as high as 3675 rads. This has also been found by other workers (Kettman and Dutton, 1971; Vann and Kettman, 1972; Hamaoka et al., 1972;

Anderson et al., 1974).

b) The anti-POL antibody response *in vivo*, a T dependent response, was removed by ^{125}I-POL suicide, showing that the POL specific B cell was eliminated by the suicide procedure.

c) Fractionation of the suicided population on Isopaque/Ficoll showed, both in *in vivo* and *in vitro* experiments, that the T cells were intact functionally following the suicide procedure. Groups which received suicided B cells, however, showed significant decreases in the anti-DNP response compared to controls. From the *in vivo* suicide experiments it appears that the IgG response to DNP is more dependent upon carried specific B cells than the IgM response. Such a result reflects the relative T cell dependence of these two antibody classes and is consistent with the notion that carrier specific B cells in some way amplify the efficiency of T help.

d) Removal of carrier specific B cells by POL rosetting and subsequent depletion on Isopaque/Ficoll yielded similar decreases in the anti-hapten response *in vitro*. Such a procedure has been previously shown to remove antigen specific B cells rather than helper cells (Elliott et al., 1973).

Suicide of T cells with ^{125}I-labelled antigen under similar conditions used in the above experiments has been reported (Roelants and Askonas, 1971; Basten et al., 1971). However, the experiments all involved transfer of suicided cells into irradiated animals and it was not known whether the radioactive antigen procedure inactivated the T cells or merely prevented them from homing to the spleen (Hamaoka et al., 1972). There have been no reports of T cell function *in vitro* being destroyed by radioactive antigen suicide. T cells from our suicided populations apparently homed to the spleen unimpeded. Furthermore, from our observations that carrier specific B cells play an important role in T-B collaboration it is clear that some of the suicide experiments reported in the literature which were believed to represent

T cell suicide may have to be re-evaluated.

From the data presented in this and the preceding chapter it is clear that interaction between T cells and carrier-specific B cells is essential if the specific T cell replacing factor is to be successfully synthesized. It is uncertain, however, whether carrier specific B cells actually produce the factor or merely present antigen to antigen-specific T cells which then secrete the factor, i.e., the cellular collaboration may be B-T-B or T-B-B. Experiments are in progress to discriminate between these two possibilities.

The chemical nature of the collaborative factor remains to be determined and until such information is available it is difficult to compare our factor with other specific thymus replacing factors already reported in the literature (Feldmann and Basten, 1972b; Feldmann et al., 1973; Gisler et al., 1973; Yu and Gordon, 1973; Taussig, 1974; Taussig and Munro, 1974). However, it was established that macrophages are required for the production of the factor but are not needed for the factor to activate B cells. The latter result is in direct contrast to the work of Feldmann who isolated a collaborative factor from activated thymus cells which was 7s IgM in nature and which had an absolute requirement for macrophages for its action (Feldmann, 1972c). However, Feldmann did not include 2-mercapto-ethanol in his cultures as we did and this compound has been shown to be able to replace at least part of the requirement for macrophages in vitro (Pierce et al., 1974). It may be that non-phagocytic cells (e.g. K cells) can also substitute in part for the presentation role of the macrophage, or that our factor and antigen can act directly on B cells, possibly utilizing the Fc receptors on B cells. It is of interest also that the small anti-θ resistant response consistently obtained was macrophage dependent, as removal of the macrophages reduced this response to background levels. Macrophages, therefore, do play a role in direct presentation of antigen to B cells. Whether this response is due to an amplification by POL specific B cells or a direct activation of DNP B cells, or both, is not known.

7.5 SUMMARY

In this Chapter it was demonstrated that carrier specific B cells play an important role in inducing an anti-hapten antibody response to the thymus-dependent antigen, DNP monomeric flagellin (DNP-MON) both *in vivo* and *in vitro*. Removal of carrier specific B cells by ^{125}I-polymeric flagellin (POL) suicide decreased the anti-IgM response by 40-50%, reduced the anti-DNP IgG response by 85-90% and completely eliminated the production of a specific T cell replacing factor described in the preceding chapter.

Several lines of evidence were presented which clearly established that the suicide procedure eliminated carrier specific B cells rather than carrier specific T (helper) cells. It was also demonstrated that macrophages were required for *production* of the specific T cell replacing factor but the *action* of the factor on B cells was macrophage independent.

CHAPTER 8

Concluding Discussion

8.1 INTRODUCTION

The experiments described in this thesis have examined some surface and functional properties of thymus-derived lymphocytes. In particular, the presence and significance of Ig on thymus cells was considered (Chapter 2) and *in vitro* techniques developed (Chapters 3, 4 and 5) to examine the role of T cells and T cell products in antibody production (Chapters 6 and 7). In this chapter an attempt is made to reconcile the results presented in this thesis with the controversial issues described in Chapter 1 and data recently reported from other laboratories.

8.2 Ig on T cells: presence and functional significance

Initially attempts were made to detect Ig on the surface of thymocytes by radioautographic and cell solubilization methods (Chapter 2). It was concluded that the majority of mouse thymus cells have <500 molecules of Ig on their surface. Only a small proportion (~1%) have greater amounts.

8.2.1 The Ig positive cells in thymus

Several other groups, in most cases using different methods of detection, have also found a small proportion of Ig positive cells in the thymus. All groups have estimated that these comprise <2% of the thymus population (Hämmerling and Rajewsky, 1971; Riethmüller and Rieber, 1972; Perkins et al., 1972; Vitetta et al., 1973). However, there have been very few attempts to characterize these cells further although it has been proposed that they may represent plasma cells, (Vitetta et al., 1973), plaque-forming cells, B cells, (Anderson and Dresser, 1972; Basten et al., 1972) or an advanced stage in the maturation of thymocytes into T lymphocytes. (Hämmerling and Rajewsky, 1971; Perkins et al., 1972; Unanue et al., 1973).

In Chapter 2 some characteristics of these cells were reported, but the major conclusions to be drawn are:-

a) they are an intrinsic part of the thymus cell population and not casual blood-borne cells or contaminants from parathymic lymph nodes.
b) the majority are not typical T or B cells, although a proportion (~20%), all lightly labelled, were

susceptible to anti-θ and complement treatment.

The functional significance of these cells was briefly considered. They do not appear to be essential for thymus cells to carry out T cell functions. Thus their removal by rosetting or by ^{125}I-anti-light chain suicide did not affect the ability of thymus cells to collaborate with B cells in an antibody response either *in vitro* or *in vivo* respectively. Hudson et al., 1974a,b showed that passage of thymus cells through anti-Ig columns does not alter the ability of the remaining cells to be "educated" to killer cells or to carry out the cytotoxic reactions. However, because of the nature of the experiments (i.e. times involved may allow development of precursor cells), definite proof that these cells do not have a role in T cell function has not been obtained. Hence exactly what these cells are and their role, if any, in the thymus is not known. It seems unlikely though, from studies of peripheral T cells that they are mature T cells about to leave the thymus. Examination of the Ig positive cells in thymus by E/M radioautography showed that they had a unique morphology not observed in spleen or lymph node (Perkins et al., 1971). It may be that they are a heterogeneous group of cells, functionally as well as morphologically, which share the common characteristic of surface Ig. Some of the more lightly labelled cells may have acquired their surface Ig cytophilically from a proportion of Ig synthesizing cells. Vitetta et al. (1973) showed that removal of cells bearing the plasma cell antigen removed the small amount of Ig synthesis that could be easily detected by thymus cell populations. Thus the fact that this 1-2% of thymus cells is an intrinsic population is relevant to reports claiming synthesis of Ig by thymus cells (Moroz and Hahn, 1973; Santana et al., 1974) and it may also have a bearing on discrepancies between total Ig detected in thymus extracts by different groups (see 1.1.1). The sensitivity of the method of detecting Ig and the size of the Ig positive population, which Basten et al., (1972) have suggested varies in different mouse strains, would influence the amounts of Ig detected.

8.2.2 Ig in thymus cell extracts

The conclusion that thymus cells do not contain large amounts of hidden Ig has been supported by most recent reports in the literature, with the notable exception of Marchalonis and coworkers, who still claim that there are large amounts of Ig on T cells (see Marchalonis and Cone, 1973; Marchalonis et al., 1974). Their objection to the microprecipitin inhibition results reported in Chapter 2 would be that the detergent NP-40 may not be effective at solubilizing the T cell membrane and releasing T cell Ig. This objection is countered to some extent by:-

a) the study of Grey et al. (1972b) who found it the most efficient method of a number examined, including freeze-thawing and urea-acetic acid (Marchalonis method) at releasing cell surface Ig; and

b) the study of Jensenius and Williams (1973) who claimed that non-ionic detergents (e.g. NP-40) led to higher recoveries of B and T cell Ig than other methods of solubilizing membranes;

c) the failure of four other groups using different systems and techniques (some similar, if not identical, to those of Marchalonis et al.), who also failed to detect large amounts of hidden Ig on thymus cells (Grey et al., 1972a; Vitetta et al., 1973; Lisowska-Bernstein et al., 1973; Jensenius and Williams, 1974). Technical reasons for the discrepancy in findings between Marchalonis and other groups have been suggested by Lisowska-Bernstein et al. (1973) and Ada and Ey (1974), but to some extent this controversy of inaccessible Ig in T cells still exists although there are now 5 negative reports to one positive.

8.2.3 Ig on peripheral T cells

As mentioned in 1.1.1, Ig appeared to be more easily demonstrated on peripheral T cells. Thus a greater proportion (>2%) of purified splenic T cells had detectable levels (by radioautography) of surface Ig compared to thymus cells. Furthermore, some functionally active splenic T cells had enough surface Ig to form Ig rosettes (Chapter 5).

However, a proportion (5-10%) of splenic T cells were shown to be able to form Fc rosettes and the possibility that the Ig positive and Fc receptor lymphocytes are one and the same cell is a likely one in view of the recent demonstrations by other workers that the Ig detected on peripheral activated T cells is mostly acquired, rather than synthesized.

Most groups did not have procedures for obtaining pure populations of splenic T cells so studies have been carried out on activated thymus cells, e.g. activated TDL, (usually activated to H-2 antigens), or T-TDL (TDL of F_1 animals given parental thymus cells), where Ig was readily demonstrated on T cells using anti-Ig reagents, although still in amounts much less than on B cells (Goldschneider and Cogen, 1973; Sprent and Hudson, 1973; Hunt and Williams, 1974; Hudson et al., 1974.) Hunt and Williams estimated there were 500-2000 molecules of Ig on "T" cells compared to 20000-150000 molecules on "B" cells in rat TDL. However, a number of lines of evidence have shown that most, if not all, of this Ig is cytophilic:-

a) There was no allelic exclusion of surface Ig (Hunt and Williams, 1974).
b) Ig, once removed by capping or trypsinization was not resynthesized (Hudson et al., 1974; Pernis et al., 1974).
c) Removal of Ig positive cells from parental thymus inoculum before injection into F_1 animals led to a decrease in Ig detected on T-TDL (Hudson et al., 1974).

Whether this is indicative that all activated thymus cells possess an Fc receptor, or whether the Ig is binding to antigen already bound on the T cell surface, as has been suggested by Sprent and Hudson (1973) and Hudson et al. (1974), is a matter of debate. However, Webb and Cooper (1973) have found that B cell Ig on peripheral circulating T cells can bind antigen, so that at least one Fab binding site is free to react. T cells have also been found to synthesize an Ig binding factor with specificity for the Fc region of IgG (Fridman and Golstein, 1974).

Hence, from the foregoing results it is clear that

both thymus and peripheral T cell populations can be contaminated with Ig of extraneous origin. Thus, for meaningful studies of T cell Ig and its synthesis and function efforts must be made to obtain purified T populations, free of B cells, and extraneous Ig removed from the T cell surface (e.g. by capping) and its resynthesis demonstrated by these purified T populations.

Thus in answer to the question posed initially in this section of the thesis - Is there Ig on T cells? - it is concluded that if there is intrinsic Ig on T cells, it is present in very much smaller amounts than on B cells, probably <500 molecules per cell. Small numbers of splenic T cells with larger amounts of Ig, and Ig detected on activated thymus cells, is most likely acquired cytophilically. The functional significance of the Ig present on T cells is still an open question. Some aspects of this question will be discussed in a later section (8.3.2.3).

8.2.4 Alternatives to Ig as the T cell receptor

A consequence of the lack of readily detectable Ig on T cells has been the proposal of alternatives to Ig as the T cell receptor. The product of the immune response (Ir) genes, genes that are genetically linked to the chief histocompatibility loci and which have been shown to influence T cell responses to a variety of antigens (see reviews: McDevitt and Benacerraf, 1969; McDevitt et al., 1971; Benacerraf and McDevitt, 1972; Grumet and McDevitt, 1973; Lieberman and Paul, 1973; McDevitt and Landy, 1973), or some other histocompatibility gene products, or complexes of the above components with Ig, have been suggested. There is mounting evidence in favour of the involvement of histocompatibility gene products in T cell recognition and function, (Crone et al., 1972; Shevach et al., 1972; Tada et al., 1973; Hämmerling and McDevitt, 1974 a,b; Taussig and Munro, 1974; Armeding and Katz, 1974; See reviews: Warner, 1974; Katz and Benacerraf, 1974), but as yet data is still too incomplete for conclusions to be made as to the nature of the T cell receptor.

8.3 The role of the T cell in cellular collaboration

8.3.1 Collaboration between T and B cells in the anti-hapten response

In the second part of this thesis characterization of *in vitro* anti-hapten responses to hapten-carrier antigens confirmed that carrier-primed lymphocytes collaborate synergistically with hapten primed lymphocytes to give an anti-hapten antibody response. The cell separation technique, described in Chapter 5, enabled a direct demonstration that carrier primed T cells and hapten specific B cells were involved in this collaboration. In addition, from a study of the anti-hapten response to DNP-MON a number of new findings were obtained. Pure populations of splenic T cells could not collaborate with B cells across a cell impermeable membrane (Chapter 6). The carrier primed B cell had a role in T-B collaboration (Chapters 3 and 6) and in particular was required for production of a specific T cell replacing factor (Chapter 7). Also, the congenitally athymic nude mouse mutant was shown to possess a small proportion of functional T cells (Chapter 4). These results have a number of implications for proposed mechanisms of T-B collaboration (1.2), the nature of the T cell receptor and results obtained by other investigators.

8.3.2 Contact vs. Factor models

From the study of the anti-hapten response, particularly to DNP-MON, the following observations relevant to mechanisms of T-B collaboration were obtained. It was shown that:-

a) Removal of T cells by anti-θ and complement treatment virtually eliminated the antibody response to DNP-MON. There was a slight residual response resistant to anti-θ treatment which could be reduced to background levels by removal of macrophages.

b) Removal of carrier specific B cells by suicide with radioactively labelled antigen or an antigen specific rosetting procedure caused a 40-50% reduction in the response.

c) Removal of macrophages also caused a 40-50% reduction in the response.

d) Pure splenic T cells, separated by the Isopaque/Ficoll procedure, could not collaborate with B cells across a cell impermeable Millipore membrane. It is noteworthy that the T cell population contained macrophages, and T cells are reportedly able to be activated by macrophages and antigen (Katz and Unanue, 1973; Klaus, 1973; Kontianen and Feldmann, 1973).

e) T and B cells and macrophages cultured together with antigen produced a specific collaborative factor which could partially (~40% restoration) restore the anti-hapten response to B cells across the Millipore membrane. The B cells did not just supply some cultural requirements for T cell activation, as it was shown that it was the carrier specific B cell in particular that was necessary for the collaborative factor to be produced. The restorations obtained were of the same order as the B cell contribution to the response (b).

f) The carrier specific B cell had a role both <u>in vitro</u> and <u>in vivo</u>, contributing to 40-50% of the IgM response and of the order of 90% to the IgG response <u>in vivo</u>.

g) Removal of macrophages abolished production of the collaborative factor.

h) Once produced macrophages were not required for the factor to restore the antibody response of B cells.

Hence the above results indicate that there is more than one mechanism of T-B collaboration. Direct interaction between antigen stimulated T and B cells, which can occur in the absence of carrier specific B cells and macrophages, and only when T and B cells are in contact, amounts to 50-60% of the IgM response to DNP-MON. It may occur either by <u>very</u> short-range factors or cell-cell contact. In addition, there appears to be a mechanism which amplifies this help phenomenon which involves macrophages and carrier specific B cells. Macrophages, carrier specific B cells and T cells in the presence of antigen allow production of a <u>specific</u> soluble factor which can activate hapten specific B cells. It is noteworthy that either carrier specific B cells, macrophages

or the T cell replacing factor contribute to a similar extent to the *in vitro* anti-DNP antibody response - i.e. approximately 50% of the total antibody response. It thus appears that the major role of macrophages in antibody formation is to aid the release of the specific T cell replacing factor. Macrophages, however, can also to a small extent stimulate B cells directly when antigen is present. This diversity of collaborative mechanisms can account for the rapidity at which an immune response is achieved and overcomes the difficulty of "rare" cells having to contact each other. Consistent with the B cell having an amplifying role in the immune response is the observation that synthesis of IgG is highly dependent upon the carrier specific B cells.

8.3.2.1 Specific collaborative factors

The preceding data does not allow a conclusion to be drawn regarding the origin of the specific collaborative factor - whether it is produced by the carrier specific B cell, or whether the carrier specific B cell is required to activate the T cell to produce it. This question is being investigated. However, it is tempting to relate the contribution of the carrier specific B cell to the phenomenon of antibody mediated enhancement of the immune response described in Chapter 1 (1.3), and to speculate that the collaborative factor from ATC populations, described by Feldmann and Basten (1972b), is also a reflection of this phenomenon, the collaborative factor being B cell Ig directed against the carrier determinant. A number of observations are consistent with this interpretation:-

a) Feldmann (1972c) characterized his specific collaborative factor as 7s IgM with specificity for the inducing antigen.

b) ATC probably possess cytophilically acquired Ig synthesized by radioresistant host B cells or by the Ig positive cells present in the thymus inoculum (see 8.2.3). As these cells have been primed *in vivo* with carrier antigen, some of the acquired antibody is probably directed against the carrier antigen.

c) Synthesis of the specific collaborative factor by ATC

has not been demonstrated. Moreover, Lisowska-Bernstein et al. (1973) showed that detectable synthesis of μ and L chains by ATC resulted from contamination of these cells by IgM containing plasma cells. Relevant to this finding is the observation that 15% of MON ATC formed Ig positive rosettes (Kirov and Parish, unpublished results). Hence it is likely that there are some carrier reactive B cells in the ATC population. Feldmann (1972c) has shown that treatment of the ATC with anti-θ and complement abolished production of the factor, but this is not sufficient to establish that it is actually produced by T cells. As shown, in Chapter 6, T cells as well as B cells were required for the production of a specific collaborative factor from spleen cells. Also, T cell metabolism may be required for release of cytophilically acquired Ig on the T cell surface, if this is the source of the factor.

d) Playfair et al. (1974) showed that activated T cells could be passively sensitized with antibody and develop co-operating properties with the specificity characteristic of the antibody. Hence collaboration via passive B cell Ig on activated T cells would appear to be a workable mechanism of collaboration.

The specific collaborative factor produced by activated thymus cells, described and characterized by another group, appears to have different properties (Taussig, 1974; Taussig and Munro, 1974). This factor was produced early in the secondary response of primed ATC to antigen in vitro (within the first 6 hours of culture). It was reported to be non-Ig in nature but was able to react with anti-histocompatibility antisera. The relationship of this factor to Feldmann's specific factor and the splenic collaborative factor (Chapters 6 and 7) remains to be established. Further characterization of the chemical nature of the splenic collaborative factor is necessary before comparisons can be made.

8.3.2.2 Non-specific collaborative factors

Non-specific collaborative factors were not really considered in experiments reported in this thesis and did not figure much in the culture system examined. However, there was some evidence that an ongoing response to DNP-MON had a facilitatory effect on the response to SRBC in the same culture (see Table 6.7). Gisler et al. (1973) found that the non-specific factor produced by activated thymus cell cultures in the presence of the priming antigen was restricted to the potentiation of antibody responses against fully or partially T independent antigens (e.g. SRBC), and that expression of the non-specific effect depended on a macrophage function which was replaced by 2ME. As 2ME was present in all cultures described in this thesis, possibly the effects of non-specific factors in the collaborative response were minimized.

8.3.3 Implications of the results for the nature of the T cell receptor

a) Demonstration that carrier specific B cells were required for production of the specific collaborative factor casts some doubt on the origin of Feldmann's specific factor. Thus the Ig nature of this factor cannot be taken as evidence that the T cell receptor is Ig (1.1.1), until there is conclusive demonstration of its synthesis by pure populations of T cells, uncontaminated by extraneous Ig.

b) Data obtained from "T" suicide experiments will have to be re-evaluated in the light of the critical role of the carrier specific B cell in collaboration. Suicide of T helper populations, in general did not cause complete abolition of the antibody response being measured (Basten et al., 1971; Roelants and Askonas 1971). In addition, in Chapter 7 it was shown that even though B cells could be readily suicided, there was no evidence that T cells were affected in function or in ability to home to the spleen *in vivo*. The possibility exists, therefore, that the decrease in antibody response observed by other groups following

suicide of T helper cell populations (probably always containing carrier specific B and T cells) was due to suicide of B cells and subsequent removal of the B cell dependent amplification mechanism. Hence, information obtained from these experiments, such as blocking of suicide with anti-Ig (Basten et al., 1971), cannot be taken as reliable indications as to the nature of the T cell receptor.

8.4 T cells in nude mice

Nude mice have been widely used as a source of pure B cell populations. Hence, the demonstration of functionally active T cells in these mice may have a bearing on many studies, particularly where responses to antigens which may be cross-reactive with environmental antigens have been examined. As an example, the results of Schrader (1973, 1974) may be considered. He found nude mouse spleen cells in culture were unable to respond to de-aggregated fowl gamma globulin (FγG). Also, they did not give a significant anti-FγG response when varying amounts (0.01 - 100 μg) POL and no FγG were added to the culture. However, when both FγG and POL were present in cultures, a significant, though low, anti-FγG response was observed. He concluded that this was evidence that substances like POL, MON and LPS - all of which gave this help effect - could substitute for T cells in giving the "second signal" to antigen activated B cells. However, an alternative and more likely explanation, is that the small proportion of nude T cells, probably well primed to these bacterial antigens, are activated by them. The resulting proliferation, possibly leading to presence of T cells specific for FγG which may allow B cell mediated enhancement of the response and cell-cell contact T help, and production of non-specific T cell replacing factors by the activated T cells, give rise to the response to FγG. (Of interest, 2ME was not present in these cultures).

The ability of nude mice to respond as well as normal mice to DNP-MON is an example of the efficiency of the amplification mechanism of the help phenomenon (8.3.2), as only a very few T cells are required to give the maximal

antibody response. Nevertheless, the T cell has a central role as shown by removal of 2-4% of spleen cells by anti-θ and complement treatment leading to a 60-70% decrease in the anti-DNP response. It also appears that the macrophage-antigen presentation mechanism in nude mice is more efficient than in normal mice, as the residual response after anti-θ treatment was consistently higher than in normal mice. It is probably through such mechanisms that nude mice are able to survive with their impaired immune system.

8.5 SUMMARY

Thus the following conclusions may be drawn from the work described in this theses:-

a) The nature of the T cell receptor is still a very open one. It can no longer be confidently assumed that it is immuno-globulin in nature, due to the small amounts of Ig present on T cells and the relatively weak evidence, mostly indirect, that Ig on T cells has functional significance.

b) There is collaboration between carrier primed and hapten specific cells in anti-hapten responses, but more than one mechanism is involved, including cell-cell contact between T and B cells, and production of specific and non-specific T cell replacing factors. The carrier specific B cell has a role in production of a specific T cell replacing factor from carrier primed spleen cells. The B cell contribution to the response amounts to 40-50% of the IgM response both *in vitro* and *in vivo* and to more than 90% of the IgG response *in vivo*, suggesting that the B cell may be involved in an amplification mechanism of the immune response. The nature of the factor and the cell responsible for its production remains to be established.

c) Nude mice possess a small proportion of functional T cells and hence cannot be regarded as pure populations of B cells, especially where responses to environmental antigens or antigens cross-reactive with environmental antigens are being considered.

SUMMARY

This thesis has dealt with some aspects of thymus-derived lymphocytes.

a) Thymus cells were examined for surface Ig by radioautography and a microprecipitin inhibition assay of detergent solubilized cell extracts. Only a small proportion (~1%) of thymus cells were found to possess significant amounts of Ig and this population was characterized. No functional significance could be attributed to these cells (Chapter 2).

b) Functional assays for T cell activity were investigated. In particular, *in vitro* anti-hapten responses to several DNP-protein conjugates were characterized (Chapter 3). It was demonstrated that congenitally athymic "nude" mice possess a small population of *functional* T cells which can collaborate in an anti-hapten response and mount a DTH reaction (Chapter 4).

c) A cell separation procedure for fractionating mouse T and B cells by rosetting and centrifugation on Isopaque/Ficoll was characterized using radioautographic techniques and the *in vitro* culture systems. It was shown by both morphological and functional criteria that pure populations of T and B cells could be obtained (Chapter 5). In addition, the procedure was modified to detect Fc receptor lymphocytes and the presence of functional T cells with Fc receptors was demonstrated.

d) Mechanisms of T-B collaboration were examined using the techniques developed in b) and c). It was established that the carrier specific B cell has a role in cellular collaboration. In *in vitro* experiments, it was shown that pure splenic T cells were unable to collaborate with B cells across a cell impermeable membrane. Carrier primed B cells were required for production of a specific collaborative factor by spleen cells. Removal of the carrier specific B cells by antigen suicide decreased the anti-DNP IgM response by 40-50% both *in vivo* and *in vitro* and reduced the IgG response by 80-90% *in vivo* (Chapters 6 and 7).

This thesis has dealt with some aspects of thymus-derived lymphocytes.

a) Thymus cells were examined for surface Ig by radioautography and a microprecipitin inhibition assay of detergent solubilized cell extracts. Only a small proportion (~1%) of thymus cells were found to possess significant amounts of Ig and this population was characterized. No functional significance could be attributed to these cells (Chapter 2).

b) Functional assays for T cell activity were investigated. In particular, *in vitro* anti-hapten responses to several DNP-protein conjugates were characterized (Chapter 3). It was demonstrated that congenitally athymic "nude" mice possess a small population of *functional* T cells which can collaborate in an anti-hapten response and mount a DTH reaction (Chapter 4).

c) A cell separation procedure for fractionating mouse T and B cells by rosetting and centrifugation on Isopaque/Ficoll was characterized using radioautographic techniques and the *in vitro* culture systems. It was shown by both morphological and functional criteria that pure populations of T and B cells could be obtained (Chapter 5). In addition, the procedure was modified to detect Fc receptor lymphocytes and the presence of functional T cells with Fc receptors was demonstrated.

d) Mechanisms of T-B collaboration were examined using the techniques developed in b) and c). It was established that the carrier specific B cell has a role in cellular collaboration. In *in vitro* experiments, it was shown that pure splenic T cells were unable to collaborate with B cells across a cell impermeable membrane. Carrier primed B cells were required for production of a specific collaborative factor by spleen cells. Removal of the carrier specific B cells by antigen suicide decreased the anti-DNP IgM response by 40-50% both *in vivo* and *in vitro* and reduced the IgG response by 80-90% *in vivo* (Chapters 6 and 7).

ACKNOWLEDGEMENTS

ACKNOWLEDGEMENTS

The work described in this thesis was carried out under a Commonwealth Postgraduate Research Award held from February 1972 to February 1975.

I am very grateful to the following people who have helped me in the course of the work:-

Professor G.L. Ada, who supervised the project,
Dr. C.R. Parish with whom some enjoyable collaborative work was done,
Ron Tha La and Keith Clarke for technical assistance,
Ralph Westen, Stuart Butterworth, Stenya Bogdanovic and the photography staff,
Richard Hill for doing the histology,
Esmée Weil and the secretaries,
and finally the many members of the JCSMR, particularly in the Department of Microbiology, who have helped to make the last few years a very pleasant and stimulating period.

BIBLIOGRAPHY

ADA, G.L., NOSSAL, G.J.V., PYE, J. & ABBOT, A., Aust. J. Exp. Biol. Med. Sci. 1964. 42 : 267.

ADA, G.L., HUMPHREY, J.H., ASKONAS, B.A., McDEVITT, H.O. & NOSSAL, G.J.V., Exp. Cell Res. 1966. 41 : 557.

ADA, G.L. & COOPER, M.G., Mechanisms of Immunological Tolerance to Non-replicating antigens, Manuscript 497, Department of Microbiology, J.C.S.M.R. 1973.

ADA, G.L. & EY, P.L., in Sela, M. (Ed.) "The Antigens" Vol. 4, Academic Press, 1974. In press.

AIRD, J., Immunology 1971. 20 : 617.

ANDERSON, H.R. & DRESSER, D.W., Eur. J. Immunol. 1972. 2 : 410.

ANDERSON, R.E., SPRENT, J. & MILLER, J.F.A.P., Eur. J. Immunol. 1974. 4 : 199.

ARMERDING, D. & KATZ, D.H., J. Exp. Med. 1974. 140 : 19.

ASKONAS, B.A., SHIMPL, A. and WECKER, E., Eur. J. Immunol. 1974. 4 : 164.

BANKHURST, A.D. & WARNER, N.L., J. Immunol. 1971. 107 : 368.

BANKHURST, A.D., WARNER, N.L. & SPRENT, J., J. Exp. Med. 1971 134 : 1005.

BANKHURST, A.D. & WARNER, N.L. Aust. J. Exp. Med. Sci. 1972. 50 : 661.

BASTEN, A., MILLER, J.F.A.P., WARNER, N.L. and PYE, J. Nature New Biol. 1971. 231 : 104.

BASTEN, A., MILLER, J.F.A.P., SPRENT, J. & PYE, J., J. Exp. Med. 1972. 135 : 610.

BASTEN, A. & HOWARD, J.G., Contemp. Top. Immunobiol. 1973. 2 : 265.

BENACERRAF, B. & McDEVITT, H.O., Science 1972. 175 : 273.

BLANDEN, R.V. & LANGMAN, R.E., Scand. J. Immunol. 1972. 1 : 379.

BLAU, J.N. & GAUGAS, J.M., Immunology 1968. 14 : 763.

BLOMGREN, H. & ANDERSSON, B., Exp. Cell. Res. 1969. 57 : 185.

BÖYUM, A. Scand. J. Clin. Lab. Invest. 1968. 21, Suppl. 97 : 77.

BRETSCHER, P.A. & COHN, M., Science, 1970. 169 : 1042.

BRETSCHER, P.A., Transplant. Rev. 1972. 11 : 217.

BRITTON, S., Scand. J. Immunol. 1972. 1 : 89.

BRODY, N.I., SISKIND, G.W. & WALKER, J.G., J. Exp. Med. 1967. 126 : 81.

BROWNSTONE, A., MITCHISON, N.A. & PITT-RIVERS, R., Immunology, 1966. 10 : 465.

BRUNNER, K.T., MAUEL, J., CEROTTINI, J.C. & CHAPUIS, B., Immunology 1968. 14 : 181.

BULLOCK, W.W. & MÖLLER, E., Transplant. Rev. 1974. 18 : 3.

BYRT, P. & ADA, G.L., Immunology, 1969. 17 : 503.

CHAPUIS, B. & BRUNNER, K.T., Int. Arch. Allergy Appl. Immunol. 1971. 40 : 321.

CHEERS, C., BREITNER, J.C.S., LITTLE, M. & MILLER, J.F.A.P. Nature New Biol. 1971. 232 : 248.

CHENG, V. & TRENTIN, J.J., Proc. Soc. Exp. Biol. Med., 1967. 126 : 467.

CLAMAN, H.N., CHAPERON, E.A. & TRIPLETT, R.F., Proc. Soc. Exp. Biol. Med. 1966. 122 : 1167.

CLAMAN, H.N. & CHAPERON, E.A., Transplant. Rev. 1969. 1 : 92.

CLAESSON, M.H., HOYER, P.E. & RØPKE, C., Scand. J. Haemat. 1969. 6 : 291.

CLINE, M.J., SPRENT, J., WARNER, N.L. & HARRIS, A.W., J. Immunol. 1972. 108 : 1126.

COHEN, J.J. & CLAMAN, H.N., J. Exp. Med. 1971. 133 : 1026.

COLE, G.J. & MORRIS, B., Aust. J. Exp. Med. Sci. 1971. 49 : 55.

COUTINHO, A., MÖLLER, G., ANDERSSON, J. & BULLOCK, W.W. Eur. J. Immunol. 1973. 3 : 299.

COOPER, M.G., Scand. J. Immunol. 1972a. 1 : 167.

COOPER, M.G., Scand. J. Immunol. 1972b. 1 : 237.

COOPER, M.G. & ADA, G.L., Scand. J. Immunol. 1972. 1 : 247.

CRONE, M., KOCH, C., & SIMONSEN, M., Transplant. Rev. 1972. 10 : 36.

CUNNINGHAM, A.J. & SZENBERG, A., Immunology, 1968. 14 : 599.

CUNNINGHAM, A.J. & SERCARZ, E.E., Eur. J. Immunol. 1971. 1 : 413.

DAIN, A.R. & HALL, J.G., Vox. Sang. 1967. 13 : 284.

DAVIES, A.J.S., LEUCHARS, E., WALLIS, V., MARCHANT, R., & ELLIOT, E.V., Transplantation, 1967. 5 : 222.

DAVIES, A.J.S., Transplant. Rev. 1969. 1 : 43.

DENNERT, G., J. Immunol. 1971. 106 : 951.

DE SOUSA, M.A.B., PARROTT, D.M.V. & PANTELOURIS, E.M., Clin. Exp. Immunol. 1969. 4 : 637.

DICKLER, H.B., ADKINSON, N.F. & TERRY, W.D., Nature, 1974. 247 : 213.

DOHERTY, P.C., ZINKERNAGEL, R.M. & RAMSHAW, I., J. Immunol. 1974a 112 : 1548.

DOHERTY, P.C. & ZINKERNAGEL, R.M., Transplant. Rev. 1974b 19 : 89.

DORIA, G., AGAROSSI, G. & DI PIETRO, S., J. Immunol. 1972. 108 : 268.

DULBECCO, R. & VOGT, M., J. Exp. Med. 1954. 99 : 167.

DUTTON, R.W., FALKOFF, R., HIRST, J.A., HOFFMANN, M., KAPPLER, J.W., KETTMAN, J.R., LESLEY, J.F. & VANN, D., Progr. Immunol. 1971. 1 : 355.

DWYER, J.M. & MACKAY, I.R., Clin. Exp. Immunol. 1972. 10 : 581.

DWYER, J.M., WARNER, N.L. & MACKAY, I.R., J. Immunol. 1972. 108 : 1439.

EISEN, H.N., in Eisen, H.N. (Ed.) Methods in Medical Research, Vol. 10, Year Book Medical Publishers, Chicago 1964, p. 94.

EKAPAHA-MENSAH, A. & KENNEDY, J.C., Nature New Biol. 1971. 233 : 174.

ELLIOTT, B.E., HASKILL, J.S. & AXELRAD, M.A. J. Exp. Med. 1973. 138 : 1133.

ESKELAND, T., KLEIN, E., INOUE, M. & JOHANSSON, B., J. Exp. Med. 1971. 134 : 265.

EY, P.L. Eur. J. Immunol. 1973. 3 : 37.

FELDMANN, M. & BASTEN, A., J. Exp. Med. 1971. 134 : 103.

FELDMANN, M., J. Exp. Med. 1972a. 135 : 735.

FELDMANN, M., J. Exp. Med. 1972b. 135 : 1049.

FELDMANN, M., J. Exp. Med. 1972c. 136 : 737.

FELDMANN, M. & BASTEN, A., Eur. J. Immunol. 1972a. 2 : 213.

FELDMANN, M. & BASTEN, A., J. Exp. Med. 1972b. 136 : 49.

FELDMANN, M. & BASTEN, A., J. Exp. Med. 1972c. 136 : 722.

FELDMANN, M. & BASTEN, A., Nature New Biol. 1972d. 237 : 13.

FELDMANN, M. & NOSSAL, G.J.V., Transplant. Rev. 1972. 13 : 3.

FELDMANN, M., WAGNER, H., BASTEN, A. & HOLMES, M., Aust. J. Exp. Biol. Med. Sci. 1972. 50 : 651.

FELDMANN, M., CONE, R.E. & MARCHALONIS, J.J., Cell. Immunol. 1973. 9 : 1.

FELDMAN, M., COHEN, I.R. & WEKERLE, H., Transplant. Rev. 1972. 12 : 57.

FLANAGAN, S.P., Genet. Res. 1966. 8 : 295.

FRIDMAN, W.H. & GOLSTEIN, P., Cell. Immunol. 1974. 11 : 442.

GARDNER, I., BOWERN, N.A. & BLANDEN, R.V., Eur. J. Immunol. 1974a. 4 : 63.

GARDNER, I., BOWERN, N.A. & BLANDEN, R.V. Eur. J. Immunol. 1974b. 4 : 68.

GEHA, R.S., SCHNEEBERGER, E., ROSEN, F.S. & MERLER, E., J. Exp. Med. 1973. 138 : 1230.

GISLER, R.H., STABER, F., RÜDE, E. & DUKOR, P., Eur. J. Immunol. 1973. 3 : 650.

GOLDSCHNEIDER, I. & COGEN, R.B., J. Exp. Med. 1973. 138 : 163.

GONATAS, N.K., ANTOINE, J-C., STIEBER, A. & AVRAMEUS, S., Lab. Invest. 1972. 26 : 253.

GORCZYNSKI, R.M., MILLER, R.G. & PHILLIPS, R.A., J. Immunol. 1972a. 108 : 547.

GORCZYNSKI, R.M., MILLER, R.G. & PHILLIPS, R.A., J. Immunol. 1972b. 110 : 968.

GORCZYNSKI, R.M., MILLER, R.G. & PHILLIPS, R.A., J. Immunol. 1973. 111 : 900.

GREAVES, M.F., TORRIGIANI, G. & ROITT, I.M., Nature, 1969. 222 : 885.

GREAVES, M.F., Transplant. Rev. 1970. 5 : 45.

GREAVES, M.F., TORRIGIANI, G. & ROITT, I.M., Clin. Exp. Immunol. 1971. 9 : 313.

GREAVES, M.F. & HOGG, N.M., Progr. Immunol. 1971. 1 : 111.

GREY, H.M., COLON, S., CAMPBELL, P. & RABELLINO, E., J. Immunol. 1972a. 109 : 776.

GREY, H.M., KUBO, R.T. & CEROTTINI, J-C., J. Exp. Med. 1972b. 136 : 1323.

GRUMET, F.C. & McDEVITT, H.O., Contemp. Top. Immunobiol. 1973. 2 : 63.

HAMAOKA, T., KATZ, D.H. & BENACERRAF, B., Proc. Nat. Acad. Sci. U.S.A. 1972. 11 : 3453.

HAMAOKA, T., OSBORNE, D.P. & KATZ, D.H. J. Exp. Med. 1973. 137 : 1393.

HÄMMERLING, U. & RAJEWSKY, K., Eur. J. Immunol. 1971. 1 : 447.

HÄMMERLING, G.J. & McDEVITT, H.O., J. Immunol. 1974a. 112 : 1726.

HÄMMERLING, G.J. & McDEVITT, H.O., J. Immunol. 1974b. 112 : 1734.

HARRIS, A.W., BANKHURST, A.D., MASON, S. & WARNER, N.L. J. Immunol. 1973. 110 : 431.

HAVAS, H.F. & HRABA, T., J. Immunol. 1969. 103 : 349.

HEMMINGSON, E.J., Int. Arch. Allergy 1972. 42 : 764.

HENRY, C. & JERNE, N.K., J. Exp. Med. 1968. 128 : 133.

HOGG, N.M. & GREAVES, M.F., Immunology 1972. 22 : 967.

HUDSON, L., SPRENT, J., MILER, J.F.A.P., PLAYFAIR, J.H.L., Nature, 1974a. 251 : 60.

HUDSON, L., GREENBERG, A.H., ROITT, I.M. & BACH, J.F., Scand. J. Immunol. 1974b. In press.

HUNT, S.V. & WILLIAMS, A.F., J. Exp. Med. 1974. 139 : 479.

IVANYI, J., SKAMENE, E. & KURISU, A., Folia Biol. (Prague) 1970. 16 : 34.

JANEWAY, C.A. Jr., J. Immunol. 1973. 111 : 1250.

JANEWAY, C.A. Jr., & PAUL, W.E., Eur. J. Immunol. 1973. 3 : 340.

JENSENIUS, J.C. & WILLIAMS, A.F., Eur. J. Immunol. 1974. 4 : 98.

KATZ, D.H., PAUL, W.E., GOIDL, E.A. & BENACERRAF, B., J. Exp. Med. 1971a. 133 : 169.

KATZ., D.H., PAUL, W.E. & BENACERRAF, B., J. Immunol. 1971b. 107 : 1319.

KATZ., D.H. & BENACERRAF, B., Adv. Immunol. 1972. 15 : 1.

KATZ, D.H., PAUL, W.E. & BENACERRAF, B. 1970. quoted in Katz, D.H. & BENACERRAF, B. Adv. Immunol. 1972. 15 : 1.

KATZ., D.H., HAMAOKA, T., DORF, M.E. & BENACERRAF, B., Proc. Nat. Acad. Sci. U.S.A. 1973a. 70 : 2624.

KATZ, D.H., HAMAOKA T., BENACERRAF., J. Exp. Med. 1973b. 137 : 1405.

KATZ, D.H. & UNANUE, E.R., J. Exp. Med. 1973. 137 : 967.

KATZ, D.H. & BENACERRAF, B., Transplant. Rev. 1974. 22 : In press.

KENNEDY, J.C., TREADWELL, P.E. & LENNOX, E.S., J. Exp. Med. 1970. 132 : 353.

KETTMAN, J. & DUTTON, R.W., Proc. Nat. Acad. Sci. U.S.A. 1971. 68 : 699.

KINDRED, B., Eur. J. Immunol. 1971. 1 : 59.

KLAUS, G.G.B., Cell. Immunol. 1974. 10 : 483.

KONTIANEN, S. & FELDMANN, M., Nature New Biol. 1973. 245 : 285.

KRETH, H.W. & WILLIAMSON, A.R., Nature, 1971. 234 : 454.

LAMELIN, J-P., LISOWSKA-BERNSTEIN, B., MATTER, A., RYSER, J.E. & VASSALLI, P., J. Exp. Med. 1972. 136 : 984.

LANGMAN, R.E., J. Immunol. Methods. 1972. 2 : 59.

LAYSON, M.N. & SEHON, A.H., Can. J. Biochem. 1967. 45 : 1773.

LECKBAND, E. & BOYSE, E.A., Science 1970. 172 : 1258.

LESLEY, J.F., KETTMAN, J.R. & DUTTON, R.W., J. Exp. Med. 1971. 134 : 618.

LIEBERMAN, R. & PAUL, W.E. Contemp. Top. Immunobiol. 1973. 3 : 117.

LISOWSKA-BERNSTEIN, B., RINUY, A. & VASSALLI, P., Proc. Nat. Acad. Sci. U.S.A. 1973. 70 : 2879.

LOOR, F. & KINDRED, B., J. Exp. Med. 1973. 138 : 1044.

LOOR, F. & ROELANTS, G.E., Nature, 1974. 251 : 229.

MARCHALONIS, J.J., CONE, R.E. & ATWELL, J.L., J. Exp. Med. 1972a. 135 : 956.

MARCHALONIS, J.J., ATWELL, J.L. & CONE, R.E., Nature New Biol. 1972b. 235 : 240.

MARCHALONIS, J.J., CONE, R.E., ATWELL, J.L. & ROLL, R.T. in Lee, J.W. & Pollak, J.K. (Eds.) The Biochemistry of Gene Expression in Higher Organisms. Aust. N.Z. Book Co., Sydney. 1972c.

MARCHALONIS, J.J. & CONE, R.E., Transplant. Rev. 1973. 14 : 3.

MARCHALONIS, J.J., CONE, R.E. & VON BOEHMER, H., Immunochemistry 1974. 11 : 271.

MASON, S. & WARNER, N.L., J. Immunol. 1970. 762 : 100.

MATTER, A., LISOWSKA-BERNSTEIN, B., RYSER, J.E., LAMELIN, J-P. & VASSALLI, P., J. Exp. Med. 1972. 136 : 1008.

McBRIDE, R.A. & SCHIERMAN, L.W., J. Exp. Med. 1970. 131 : 377.

McBRIDE, R.A. & SCHIERMAN, L.W., J. Immunol. 1973. 110 : 1710.

McDEVITT, H.O. & BENACERRAF, B., Adv. Immunol. 1969. 11 : 31.

McDEVITT, H.O., BECHTOL, K.B., GRUMET, F.C., MITCHELL, G.F. & WEGMANN, T.G. Progr. Immunol. 1971. 1 : 495.

McDEVITT, H.O. & LANDY, M. Eds. Genetic Control of Immune Responsiveness. Acad. Press., N.Y. 1973.

MILLER, J.F.A.P. & MITCHELL, G.F., J. Exp. Med. 1968. 128 : 801.

MILLER, J.F.A.P. & MITCHELL, G.F., Transplant. Rev. 1969. 1 : 3.

MILLER, J.F.A.P., BASTEN, A., SPRENT, J. & CHEERS, C. Cell. Immunol. 1971a. 2 : 469.

MILLER, J.F.A.P., SPRENT, J., BASTEN, A., WARNER, N.L., BREITNER, J.C.S., ROWLAND, G., HAMILTON, J., SILVER, H. & MARTIN, W.J., J. Exp. Med. 1971b. 134 : 1266.

MILLER, J.F.A.P., Int. Rev. Cytol. 1972. 33 : 77.

MITCHELL, G.F., Contemp. Topics Immunobiol. 1974. 3 : 97.

MITCHISON, N.A. in : Landy, M. & Braun, W. (Eds.) Immunological tolerance, Academic Press, New York and London 1969. p. 115.

MITCHISON, N.A., RAJEWSKY, K., TAYLOR, R.B., in Sterzl, J. & Rhia, I. (Eds.) Developmental Aspects of Antibody Formation and Structure, Vol. 2, Prague Symp., Academic Press, New York, 1970. p. 547.

MITCHISON, N.A., Eur. J. Immunol. 1971. 1 : 10.

MODABBER, F. & COONS, A.H., J. Immunol. 1972. 108 : 1447.

MÖLLER, G., Cell. Immunol. 1970. 1 : 573.

MOND, J.J., TAKAHASHI, T. & THORBECKE, G.J., J. Exp. Med. 1972. 136 : 663.

MOORE, C.H., HENDERSON, R.W. & NICHOLL, L.W., Biochemistry 1968. 7 : 4075.

MORRIS, B., Contemporary Topics in Immunology 1973. 2 : 39.

MOROZ, C. & HAHN, Y., Proc. Nat. Acad. Sci. U.S.A. 1973. 70 : 3716.

MURGITA, R.A. & VAS, S.I., Immunology 1972. 22 : 319.

NIEDERHUBER, J.E. & MÖLLER, E., Cell. Immunol. 1973. 6 : 407.

NOBLE, P.B., CUTTS, J.H., CARROLL, K.K., Blood 1968. 31 : 66.

NOSSAL, G.J.V., CUNNINGHAM, A., MITCHELL, G.F. & MILLER, J.F.A.P., J. Exp. Med. 1968. 128 : 839.

NOSSAL, G.J.V., WARNER, N.L., LEWIS, H. & SPRENT, J., J. Exp. Med. 1972. 135 : 405.

ORR, K.B. & PARASKEVAS, F., J. Immunol. 1973. 110 : 456.

PARISH, C.R. & STANLEY, P., Immunochemistry 1972. 9 : 853.

PARISH, C.R. & HAYWARD, J.A., Proc. R. Soc. Lond. B. 1974a. 187 : 47.

PARISH, C.R. & HAYWARD, J.A., Proc. R. Soc. London. B. 1974b. 187 : 65.

PARISH, C.R. & HAYWARD, J.A., Proc. R. Soc. Lond. B. 1974_c. In press.

PERKINS, W.D., KARNOVSKY, M.J. & UNANUE, E.R., J. Exp. Med. 1972. 135 : 267.

PERNIS, B., FORNI, L. & AMANTE, L., J. Exp. Med. 1970. 132 : 1001.

PERNIS, B., MILLER, J.F.A.P., FORNI, L. & SPRENT, J., Cell. Immunol. 1974. 10 : 476.

PIERCE, C.W., KAPP, J.A., WOOD, D.D. & BENACERRAF, B., J. Immunol. 1974. 112 : 1181.

PILARSKI, L.M. & CUNNINGHAM, A.J. J. Immunol. 1974. In Press.

PINCUS, C., MILLER, G. & NUSSENZWEIG, V., J. Exp. Med. 1971. 133 : 987.

PINCUS, C., MILLER, G. & NUSSENZWEIG, V., J. Immunol. 1973. 110 : 301.

PLAYFAIR, J.H.L., Clin. Exp. Immunol. 1971. 8 : 839.

PLAYFAIR, J.H.L., Clin. Exp. Immunol. 1974. 17 : 1.

PLAYFAIR, J.H.L., MARSHALL-CLARKE, S. & HUDSON L., Eur. J. Immunol. 1974. 4 : 54.

PORTER, R.R., Biochem. J. 1959. 73 : 119.

RABELLINO, E., COLON, S., GREY, H.M. & UNANUE, E.R., J. Exp. Med. 1971. 133 : 156.

RAFF, M.C., Immunology 1970a. 19 : 637.

RAFF, M.C., Nature 1970b. 226 : 1257.

RAFF, M.C. & WORTIS, H.H., Immunology 1970. 18 : 931.

RAFF, M.C., STEINBERG, M. & TAYLOR, R.B., Nature 1970. 225 : 553.

RAFF, M.C. & CANTOR, H., Progr. Immunol. 1971. 1 : 83.

RAJEWSKY, K., ROTTLANDER, E., PELTRE, G. & MILLER, B. J. Exp. Med. 1967. 126 : 581.

RAJEWSKY, K., ROELANTS, G.E. & ASKONAS, B.A., Eur. J. Immunol. 1972. 2 : 592.

RIETHMÜLLER, G. & RIEBER, E.P., Progr. Immunol. 1971. 1 : 127.

ROELANTS, G.E. & ASKONAS, B.A., Eur. J. Immunol. 1971. 1 : 151.

ROELANTS, G.E., RYDÉN, A., HÄGG, L-B. & LOOR, F., Nature 1974. 247 : 106.

ROSENTHAL, M., STASTNY, P. & ZIFF, M., J. Immunol. 1973. 111 : 1119.

ROUSE, B.T. & WARNER, N.L., Cell. Immunol. 1972. 3 : 470.

RUBIN, B., Scand. J. Immunol. 1972a. 1 : 125.

RUBIN, B., Scand. J. Immunol. 1972b. 1 : 135.

RUBIN, A.S. & COONS, A.H., J. Immunol. 1972a. 108 : 1597.

RUBIN, A.S. & COONS, A.H., J. Exp. Med. 1972b. 136 : 1501.

RUBIN, A.S., MacDONALD, A.B. & COONS, A.H., J. Immunol. 1973. 111 : 1314.

RUSSEL, E.S. & BERNSTEIN, S.E., in Green, E.L. (Ed.), Biology of the Laboratory Mouse, 2nd edition McGraw Hill, New York, 1966. p. 352.

RYGAARD, J. & POVLSEN, C.O., Acta Pathol. Microbiol. Scand. 1969. 77 : 758.

RYGAARD, J., Acta Pathol. Microbiol. Scand. 1969. 77 : 761.

SANTANA, V., WEDDERBURN, N. & TURK, J.L., Immunology 1974. 27 : 65.

SANTER, V., BANKHURST, A.D. & NOSSAL, G.J.V., Exp. Cell. Res. 1972. 72 : 377.

SCHIERMAN, L.W., LECKBAND, E. & McBRIDE, R.A., Proc. Soc. Exp. Biol. Med. 1969. 130 : 744.

SCHIMPL, A. & WECKER, E., Nature New Biol. 1972. 237 : 15.

SCHIMPL, A. & WECKER, E., J. Exp. Med. 1973. 137 : 547.

SCHRADER, J.W., J. Exp. Med. 1973. 137 : 844.

SCHRADER, J.W., Eur. J. Immunol. 1974. 4 : 20.

SHEVACH, E., PAUL, W.E. & GREEN, I., J. Exp. Med. 1972. 136 : 1207.

SILVER, H., MILLER, J.F.A.P. & WARNER, N.L., Int. Arch. Allergy 1971. 40 : 540.

SJÖBERG, O., ANDERSSON, J. & MÖLLER, G., J. Immunol. 1972. 109 : 1379.

SPRENT, J. & HUDSON, L., Transplant. Proc. 1973. V : 1731.

STRAUSBAUCH, P., SULICA, A. & GIVOL, D., Nature, 1970. 227 : 68.

TADA, T., OKUMURA, K. & TANIGUCHI, M., J. Immunol. 1973. 111 : 952.

TAUSSIG, M.J. & LACHMANN, P.J., Immunology 1972. 22 : 185.

TAUSSIG, M.J., Nature, 1974. 248 : 234.

TAUSSIG, M.J. & MUNRO, A.J., Nature, 1974. 251 : 63.

TAYLOR, R.B., Nature, 1968. 220 : 611.

TERRES, G. & MORRISON, S.L., J. Immunol. 1967. 98 : 584.

THEIS, G.A. & THORBECKE, G.J., J. Immunol. 1972. 110 : 91.

TIKASINGH, E.S., SPENCE, L. & DOWNS, W.G., Amer. J. Trop. Med. Hyg. 1966. 15 : 291.

TILL, J.E. & McCULLOCH, E.A., Rad. Res. 1961. 14 : 213.

TRUMP, G.N., J. Immunol. 1972. 109 : 754.

UNANUE, E.R., J. Immunol. 1970. 105 : 1339.

UNANUE, E.R., GREY, H.M., RABELLINO, E., CAMPBELL, P. & SCHMIDTKE, J., J. Exp. Med. 1971. 133 : 1188.

UNANUE, E.R., ENGERS, H.D. & KARNOVSKY, M.J., Fed. Proc., Fed. Amer. Soc. Exp. Biol. 1973. 32 : 44.

VANN, D.C. & KETTMAN, J.R., J. Immunol. 1972. 108 : 73.

VITETTA, E.S., BIANCO, C., NUSSENZWEIG, V. & UHR, J.W., J. Exp. Med. 1972. 136 : 81.

VITETTA, E.S., UHR, J.W., BOYSE, E.A., Proc. Nat. Acad. Sci. U.S.A. 1973. 70 : 834.

WALDMANN, H. & MUNRO, A., Nature 1973. 243 : 356.

WALDMANN, H. MUNRO, A. & HUNTER, P., Eur. J. Immunol. 1973. 3 : 167.

WALDMANN, H. & MUNRO, A., Immunology 1974. 27 : 53.

WALKER, J.G. & SISKIND, G.W., Immunology 1968. 14 : 21.

WARNER, N.L., Aust. J. Exp. Biol. Med. Sci. 1964. 42 : 401.

WARNER, N.L., Adv. Immunol. 1974. 19 : 67.

WATSON, J., J. Immunol. 1973. 111 : 1301.

WEBB, S.R. & COOPER, M.D., J. Immunol. 1973. 111 : 275.

WIGZELL, H., GOLSTEIN, P., SVEDMYR, E.A.J. & JONDAHL, M., Transplant. Proc. 1972. IV : 311.

WORTIS, H.H., Clin. Exp. Immunol. 1971. 8 : 305.

YAMADA, H. & YAMADA, A., J. Immunol. 1969. 103 : 357.

YOSHIDA, T.O & ANDERSSON, B., Scand. J. Immunol. 1972. 1 : 401.

YU, H. & GORDON, J., Nature New Biol. 1973. 244 : 20.

ZINKERNAGEL, R.M. & DOHERTY, P.C., Nature 1974. 251 : 547.